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RESEARCH PAPER

# Newly isolated *Bacillus* sp. G51 from Patagonian wool produces an enzyme combination suitable for felt-resist treatments of organic wool

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**Abstract** Bacteria from Patagonian Merino wool were isolated to assess their wool-keratinolytic activity and potential for felt-resist treatments. Strains from *Bacillus, Exiguobacterium, Deinococcus*, and *Micrococcus* produced wool-degrading enzymes. *Bacillus* sp. G51 showed the highest wool-keratinolytic activity. LC-MS/MS analysis revealed that G51 secreted two serine proteases belonging to the peptidase family S8 (MEROPS) and a metalloprotease associated with Bacillolysin, along with other enzymes (γ-glutamyltranspeptidase and dihydrolipoyl dehydrogenases) that could be involved in reduction of keratin disulfide bonds. Optimum pH and temperature of G51 proteolytic activity were 9 and 60 °C, respectively. More than 80% of activity was retained in $H_2O_2$, Triton X-100, Tween 20, Lipocol OXO650, Teridol B, and β-mercaptoethanol. Treatment of wool top with G51 enzyme extract caused a decrease in wool felting tendency without significant weight loss (<1.5%). Sparse work has so far been performed to investigate suitable keratinases for the organic wool sector. This eco-friendly treatment based on a new enzyme combination produced by a wild bacterium has potential for meeting the demands of organic wool processing which bans the use of hazardous chemicals and genetic engineering.

**Keywords** Organic wool · Felting · Keratinases · *Bacillus* · Patagonia

## Introduction

Wool is an animal hair fiber that consists mainly on proteins (up to 90–95% of the fiber) of two major types, the intermediate filament forming keratin proteins and the keratin associated proteins [1]. The wool surface is formed by overlapping hydrophobic cuticle scales that cover the cortex cells in the interior of the fiber [2]. During aqueous washing, the scale structure of the cuticle and the associated differential friction effect lead to a progressive entanglement of the wool fiber, resulting in felting shrinkage [3]. The commercial most common process for imparting shrink resistance to wool is the chlorine-Hercosett method which combines acid chlorination followed by a cationic polyamide epichlorhydrin polymer application [4]. Drawbacks of this process may include yellowing of wool, fiber strength loss, poor handling quality, and harmful effluents containing absorbable organic halogens (AOX) [5]. Thus, one of the main need is the development of environmentally friendly felt-resist treatments which permit wool washing in domestic machines. This is even more important for the organic wool sector which requires processes with low environmental impacts. Ladwig et al. [6] reported that, globally, approximately 1% of the sheep are organically managed with a total flock in 2013 of almost 12 million, which could achieve a 10–15% premium over non-organic prices.

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The improvement of wool fiber attributes could contribute to the valorization of organic wool, expanding their opportunities for growth in the global wool market. The Global Organic Textile Standard (GOTS) defines the world processing standard for textiles made from organic fibers, including wool. Chlorination of wool is prohibited by GOTS [7] due to the individual health and environmental risks. Enzymatic shrink-resist treatments arose among chlorine-free processes because proteases with keratinolytic activity can be used for wool descaling to improve shrink resistance [8]. Cegarra et al. [9] modeled the action of the enzyme Novolan T (Novo Nordisk), produced by a genetically modified *Bacillus* strain, on the physical characteristics of a wool fabric and determined the optimal treatment conditions. Other biocatalysts, Bactosol WO (Sandoz Chemikalien AG) and Savinase/Novozym (Novo Nordisk), had a positive influence on wool fiber properties inducing a decrease in shrinkage [10]. Lenting et al. [11] showed that a pre-treatment with hydrogen peroxide and high salt concentration modify specifically the outer surface protein layer of wool making this fiber susceptible for proteolytic hydrolysis, which resulted in shrink resistance with a limited impact on tensile strength loss. To restrict protease activity to the wool cuticle and avoid an excessive damage of the fiber, different approaches such as chemical and genetical modification of proteases have proved to be useful [12–14]. For example, wool anti-felting effect and improved control on enzyme activity were reached using Esperase covalently linked onto Eudragit S100 [13]. A recombinant high molecular weight protease, subtilisinE-VPAVG$_{220}$, produced a significant reduction of pilling, weight loss, and tensile strength loss of wool fibers in comparison with Esperase (Sigma–Aldrich) [12]. Another recombinant protease proposed to prevent wool shrinkage was an extreme alkaline, oxidation-resistant keratinase from *Bacillus licheniformis* BBE11-1 expressed in *Bacillus subtilis* WB600 [15].

Despite the number of studies about application of keratinases to reduce wool-shrink tendency, sparse work has so far been performed to investigate suitable keratinases for organic wool processing. Besides rejecting wool chlorination, GOTS prohibits the use of enzymes derived from genetically modified organisms in textile processing [7]. Nowadays, genetically modified (engineered) microorganisms (GMM) are generally used to produce commercial Subtilisins, to enhance productivity or to introduce desired traits such as stability enhancement [16]. Most of the proteases investigated to date for improving wool shrink-resistance are commercial Subtilisins or enzymes derived from GMM. The isolation and screening of wild keratinolytic strains from natural environments or wool sheep could, therefore, open new opportunities for the development of felt-resist treatments of organic wool based on novel keratinases. Accordingly, the aim of this study was to isolate native bacteria from Patagonian Merino wool to characterize their keratinolytic enzymes and to assess their potential for felt-resist treatments suitable for organic wool processing.

## Materials and methods

### Isolation of protease-producing bacteria

Samples of raw Merino wool were collected from 5 sheep shearing zones in Patagonia (Argentina): Río Negro Province (Ñorquinco), Tierra del Fuego Province, and Chubut Province (Meseta Central and two sheep breeding ranches near to Puerto Madryn city). To enhance the possibilities of isolating protease-producing microorganisms, the samples were processed by two methods: direct platting on Skim milk (SM) and Humic acid-vitamin (HAV) agar, and protease-producing microorganism enrichment and platting on SM agar. Thus, each wool sample was divided in two subsamples, one of them suspended in sterile saline solution was used to spread SM and HAV agar plates. SM agar contained (g/L in distilled water) skim milk 12.5, yeast extract 3, NaCl 5, agar–agar 16 (pH 7.8). HAV agar was prepared according to Hayakawa et al. [17]. The second wool subsample was incubated in a mineral salt medium MSM (g/L in distilled water $K_2HPO_4{\cdot}3H_2O$ 1.5; $MgSO_4{\cdot}7H_2O$ 0.1; $CaCl_2$ 0.1; $FeSO_4{\cdot}7H_2O$ 0.03; $ZnSO_4{\cdot}7H_2O$ 0.005) at 30 °C and 200 rpm for 5 days. In this medium, wool was the only source of carbon and nitrogen which favors the development of microorganisms that release extracellular proteases to depolymerize keratin [18]. Microorganisms grown in both HAV agar and MSM were sub-cultured on SM agar to detect protease activity. The SM agar plates were incubated at 30 °C and observed daily for signs of clearing of the agar around the colonies during 5 days. The proteolytic isolates were purified by repeated streaking in the same medium and stored at −80 °C using 20% (v/v) glycerol as cryoprotectant.

### Screening of protease-producing bacteria

To select the isolates with the higher protease activity, a spot technique on SM agar was used [19]. For each isolate, three 100 mL Erlenmeyer flasks containing 30 mL of SM liquid medium were inoculated with an overnight culture at 5% (v/v) ratio and incubated at 30 °C and 200 rpm in an orbital shaker for 72 h. The cultures were centrifuged (4 °C, 16,200*g*, 10 min) and the supernatants (5 µL) were placed on SM agar plates. Halos generated by proteolytic activity were recorded every day for a 3 day-period at 30 °C. The

strains showing a clearance diameter >0.5 cm at day 1 and >1 cm at day 3 were selected for further studies.

### 16S rDNA amplification and sequencing

DNA from isolates was extracted using the Wizard Genomic DNA Purification kit (Promega Corporation, Madison, USA). In a first step, almost 500 bp of the 16S rRNA gene of the isolates was sequenced using the primers 27f and 518r. For the selected isolate G51, the 16S rRNA gene sequence (corresponding to positions 27–1492 in the *Escherichia coli* gene) was PCR-amplified as described in Olivera et al. [20], using a Multigene Gradient thermal cycler (Labnet International Inc., Woodbridge, USA). Sequencing on both strands of PCR-amplified fragments was performed using the dideoxy chain termination method by the commercial services of IDEGEN (Argentina). The 16S rRNA gene sequences were compared to the GenBank nucleotide database with BLAST and identified using the EzTaxon-e program [21]. Sequences were deposited at the GenBank database under the accession numbers KX353764-KX353773.

### Keratinolytic activity

Keratinolytic activity was examined using wool top as substrate. Wool fibers were cut into small fragments, ground well, and passed through a small-mesh grid to remove coarse particles [22]. One milliliter of culture supernatant (see "Screening of protease-producing bacteria") was incubated with 1% (w/v) milled wool (1 mL), in 50 mM TRIS–HCl buffer (pH 8.0) at 45 °C and 100 rpm for 2 h. The reaction was terminated with 2 mL of 10% (w/v) trichloroacetic acid (TCA) and then, the reactants were allowed to stand for 30 min at 4 °C. Controls were treated the same way except that TCA was added before the incubation. After centrifugation (10,000*g*, 10 min), 1 mL of the supernatant was added with 5 mL of 0.5 M $Na_2CO_3$ and 1 mL of Folin–Ciocalteu reagent, and then the mixture was incubated at 37 °C for 30 min [23]. The keratinolytic activity was measured at 660 nm with a JENWAY 6320D visible spectrophotometer (Dunmow, Essex, UK). The data presented are mean values of three independent determinations. One keratinolytic activity unit (KAU) was defined as the amount of enzyme which liberates 1 µmol tyrosine/min under the assay conditions.

### Caseinolytic Activity

The reaction mixture contained 1.1 mL of 1% (w/v) casein (SIGMA) in 0.1 M TRIS–HCl buffer (pH 8.0) and 0.1 mL of enzyme extract. The reaction was carried out at 45 °C and stopped by the addition of 5% (w/v) TCA (1.8 mL). The controls were treated the same way except that TCA was added before the incubation. The reactants were allowed to stand at 4 °C for 30 min, and centrifuged (10,000*g*, 10 min). Caseinolytic activity was then determined spectrophotometrically using the Folin–Ciocalteu method [23]. The results were calculated as mean values of three independent replicas. One caseinolytic activity unit (CAU) was defined as the amount of enzyme which liberates 1 µmol tyrosine/min under the assay conditions.

### Characterization of the proteolytic activity of isolate G51

#### *Influence of temperature, pH, and chemical agents on proteolytic activity*

The proteolytic activity of the cell-free supernatant of the selected isolate G51 was characterized. A range of temperatures between 30 and 70°C were used for determining the optimum temperature of caseinolytic activity. Similarity, optimum pH was determined by assaying different pH buffers [0.067 M $KH_2PO_4$–$Na_2HPO_4$ (pH 6–7), 0.2 M TRIS–HCl (pH 8–9), 0.2 M $Na_2CO_3$–$NaHCO_3$ (pH 10)]. Thermostability was investigated by pre-incubating G51 supernatant without substrate at 40, 50, 60, and 70 °C from 0 to 120 min, before measuring the residual caseinolytic activity. To determine pH stability, the cell-free supernatant was precipitated with three volumes of cold acetone (−20 °C), centrifuged (4 °C, 16,200*g*, 10 min), and re-dissolved in the same initial volume of Good´s buffers (pH 6–10) [24] and 50 mM $Na_2HPO_4$-NaOH buffer (pH 11). The pH stability (residual caseinolytic activity) was determined after left the samples on ice for 120 min. Caseinolytic activity at minute zero was considered as 100% residual activity. The effects of $Ca^{2+}$ and $Zn^{2+}$ (5 mM $CaCl_2$ and 5 mM $ZnSO_4$), surfactants (1 and 5% v/v Tween 20, 1 and 5% v/v Triton X-100, 1% w/v SDS), surfactants/emulsifiers used in the wool industry (1% v/v Lipocol OXO 650 and Teridol B), protease inhibitors (10 mM 1,10-phenanthroline, 0.1 mM E64, 1–10 mM PMSF, 0.1 mM iodoacetamide, 10 mM EDTA), and reducing /oxidizing agents (5 mM β-mercaptoethanol, 5 mM DTT, 1% $H_2O_2$) on caseinolytic activity were investigated after pre-incubating the G51 cell-free supernatant with such chemicals at 30 °C for 1 h. Controls were prepared by pre-incubating the samples with the appropriate solvents used to dissolve the chemicals assayed. The results were calculated as mean values of three independent replicas.

#### *SDS–PAGE/zymographic assay*

G51 cell-free supernatant (400 µL) was precipitated with three volumes of cold acetone (−20 °C) with gentle

agitation. The suspension was settled at −20 °C for 1 h, and then it was centrifuged (4 °C, 10,000*g*, 10 min). The pellet was dried under vacuum and it was re-dissolved in 50 µL of 65 mM TRIS–HCl (pH 6.8) containing 2% (w/v) SDS, 8% glycerol, and 0.002% (w/v) bromophenol blue (protein content: 1.25 mg/mL). SDS–PAGE (10.0%) was performed in a Mini-Protean II Dual Slab Cell (BioRad, Hercules, USA) at constant voltage (200 volts) for 60 min. Resolution buffer contained 3 M TRIS–HCl (pH 8.8) with 1% (w/v) SDS, and reservoir buffer 25 mM TRIS–HCl (pH 8.3) with 0.192 M glycine and 0.1% (w/v) SDS. Samples were loaded together with a molecular weight marker (Low Range, Bio-Rad). Proteins were detected by colloidal staining with Coomassie brilliant blue G-250 [25]. Zymographic analysis was performed according to Westergaard et al. [26].

*Analysis of proteins by LC-MS/MS*

Detectable SDS–PAGE protein bands which showed proteolytic activity in zymographic analysis were excised from the gel. Protein digestion and Mass Spectrometry analysis (LC-MS/MS) were performed at the Proteomics Core Facility CEQUIBIEM, at the University of Buenos Aires/CONICET as follows: excised protein bands were sequentially washed with 50 mM ammonium bicarbonate, 25 mM ammonium bicarbonate 50% acetonitrile, and 100% acetonitrile; reduced and alkylated with 10 mM DTT and 20 mM iodoacetamide and in-gel digested with 120 ng of trypsin in 25 mM ammonium bicarbonate at 37 °C overnight. Peptides were recovered by elution with 50% acetonitrile-0.5% trifluroacetic acid, including brief sonication, and further concentrated by speed-vacuum drying. Samples were resuspended in 15 µL of water containing 0.1% formic acid and desalted using C18 zip tips (Merck Millipore) and eluted in 10 µL of $H_2O$:ACN:FA 40:60:0.1%.

The digests were analyzed by nanoLC-MS/MS in a Thermo Scientific Q Exactive Mass Spectrometer (Thermo Scientific, Bremen, Germany). A 120 min gradient of $H_2O$:ACN at a flow of 300 nL/min was used with a C18 2 mm Easy Spray column × 150 mm. Data Dependent MS2 method was used to fragment the top 12 peaks in each cycle. Mass spectrometry data were first analyzed by generating .msf files from raw MS and MS/MS spectra using the Proteome Discoverer 1.4 software (Thermo Fisher Scientific) and the database searches were then performed using the SEQUEST search engine, against the *Bacillus subtilis* Uniprot protein sequence database. The following search parameters were applied; enzyme: trypsin (full); missed cleavage sites, 2; precursor mass tolerance, 10 ppm; fragment mass tolerance: 0.05 Da; Dynamic modifications: oxidation (M); fixed modifications: carbamidomethyl (C). Proteins were considered as significant hits if the following conditions were met: false discovery rate (FDR) less than 1%; identified by at least two different high confidence peptides.

**Treatment of wool fibers using proteases from isolate G51**

*Analysis of wool surface modification*

Merino wool tops used in these studies were kindly supplied by Fuhrmann S.A. (Trelew, Argentina). To evaluate whether isolate G51 proteases could be used to degrade wool cuticle, 200 mg of wool top fibers (19.4 µm) were soaked in a solution consisting on 10 mL of G51 free-cell supernatant and 10 mL of 50 mM TRIS–HCl buffer (pH 8.0) with gentle shaking (50 rpm) at 50 °C for 2 h. Then, wool fibers were washed extensively with distilled water and air dried. Controls using Esperase 8.0 L (SIGMA) and without enzymes (only with buffer) were performed. The final concentration of G51 proteases and Esperase was approximately 1.5 CAU/mL of the bath. Aliquots of the wool fibers were subjected to scanning electron microscopy (SEM) to identify any surface modifications using a JEOL JSM 6460LV scanning electron microscope (JEOL, Tokyo, Japan) with an accelerating voltage of 15 kV.

*Enzymatic felt-resist treatment*

G51 enzyme extract was prepared by precipitation of the cell-free supernatant with three volumes of cold acetone (−20 °C). After centrifugation (4 °C, 16,200*g*, 10 min), the pellet was re-dissolved in 50 mM TRIS–HCl buffer (pH 8.0). Approximately 1.1 g of wool top (23.5 µm) was disposed in a bath (mass:liquid ratio 1/20) containing 50 mM TRIS–HCl buffer (pH 8.0) with 5 mM $CaCl_2$. The enzyme concentration of G51 proteases or Esperase 8.0 L (SIGMA) was 7.5 CAU/mL of the bath. Controls without enzymes were also performed. Five independent replicas of each treatment were processed. Treatments were conducted at 45 °C and 30 rpm for 1 h. After incubation, wool fibers were removed, washed thoroughly, and air-dried. Thereafter, wool fibers were kept at 50 °C for 2 h, desiccated, and weighted until constant weight (considered as differences between successive weights inferior to 1 mg) [12]. Loose wool feltability was determined using a modified Aachen felt test [27]. Felt-ball diameter was measured at three places and the values averaged, on five felt-ball independent replicas for each treatment. Then, the felt-ball density was calculated (IWTO-20-04). An increase in felt-ball diameter, and consequently a decrease of felt-ball density, indicates a smaller felting propensity (and vice versa). The significance of the differences in felt-ball diameter and density among treatments was evaluated by one-way ANOVA. Tukey was used for multiple comparisons. As

the percentage of weight loss between control and protease treatments did not accomplish ANOVA assumptions, the Mann–Whitney test was performed. Statistical analyses were carried out with SPSS 7.0 package [28].

## Results and discussion

### Screening and selection of protease-producing bacteria from Patagonian Merino wool

A lot of effort has been concentrated on the development of environmental acceptable felt-resist treatments for wool, mainly using commercial proteases or their modifications [10, 11, 13, 14]. The bioprospection of microbial diversity might also provide keratinases or natural mixtures of them with interesting properties for organic wool processing [7]. From 135 bacterial isolates of Patagonian Merino wool, 66 showed proteolytic activity (23 were isolated on SM agar, 20 on HAV agar, and 23 after enrichment in MSM). Figure 1a, b shows the diameters of the halos of culture supernatants of the isolates with higher proteolytic activity, obtained by the spot technique on SM agar, after 1 and 3 days of incubation. Culture supernatants of most of such isolates also presented keratinolytic activity on Merino wool (Fig. 1c). From the isolates with higher keratinolytic activity, 7 corresponded to the genus *Bacillus* (G11, G21, G40, G49, G51, H23, and H36) and only one

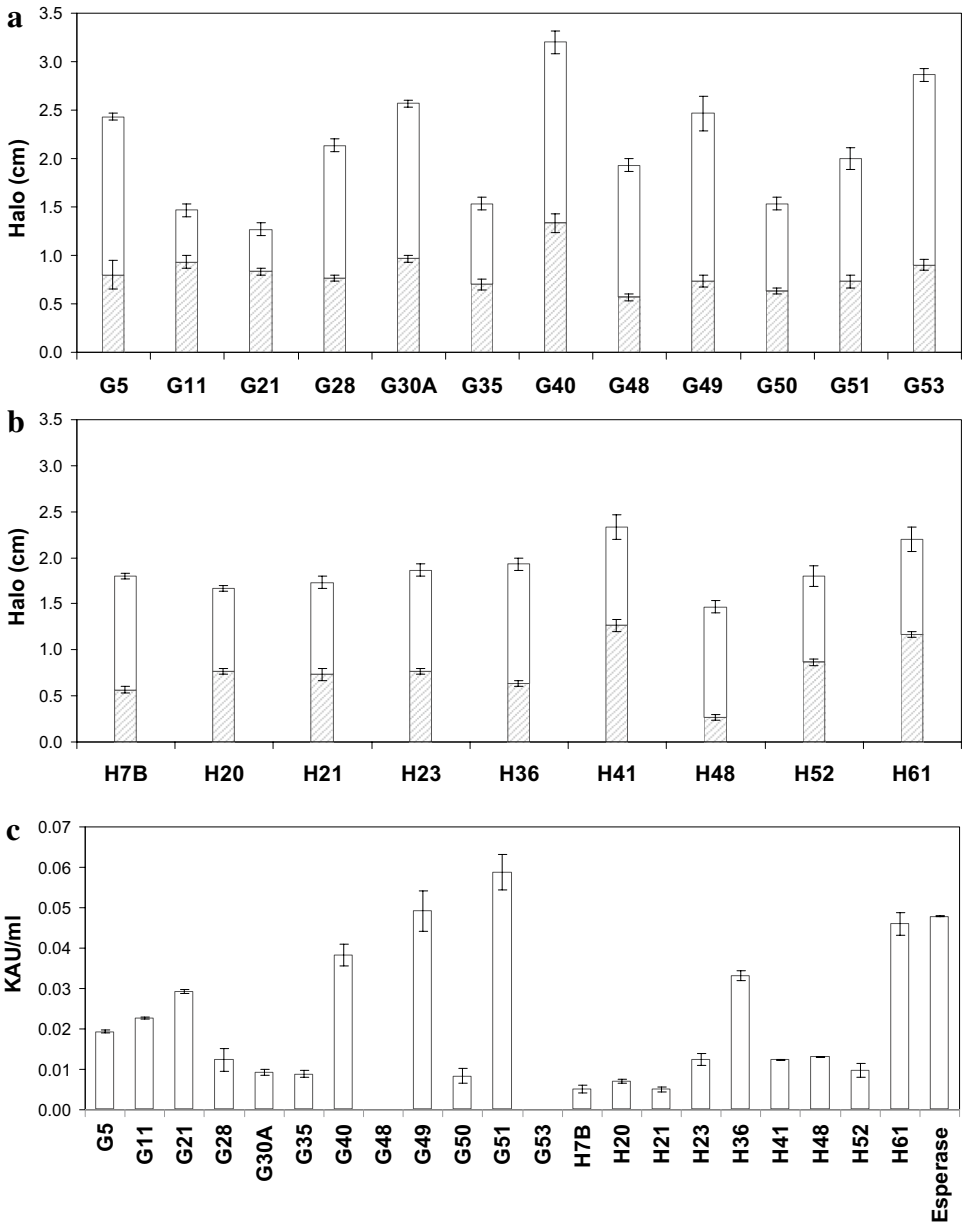


**Fig. 1** Diameters of the halos produced by spots (5 μL) of culture supernatants of Patagonian Merino isolates on SM agar plates after 1 (*grey part of the bars*) and 3 days of incubation (*grey plus white parts of the bars*). **a** Strains isolated from wool samples incubated in MSM medium (G11, G21, G35, G40, G53) and on SM agar (rest of the isolates); **b** strains isolated on HAV agar; **c** keratinolytic activity of culture supernatants of isolates from Patagonian Merino wool. Data: means ($n = 3$) ± 1 SE

to each of the genera *Exiguobacterium* (G5), *Deinococcus* (H48), and *Micrococcus* (H61), (Online Resource 1). Few studies have screened wool bacteria as a source of keratinolytic enzymes, thus the knowledge about its usefulness for wool processing is limited. *Bacillus* sp. strain HTS 102, isolated from Portuguese autochthonous sheep, produces a strong and stable protease with keratinolytic activity [19]. Infante et al. [29] isolated *Bacillus thuringiensis* L11 from Merino wool which produces an extracellular protease with potential for the textile industry. In this study, isolate G51 was selected for enzymatic treatment of wool as it showed the highest wool keratinolytic activity (Fig. 1c). G51 16S rRNA gene sequence (~1,400 bp) shared 99.93% similarity with that of *Bacillus subtilis* sp. *inaquosorum* 13429$^T$, a GRAS (generally recognized as safe) species with valuable biotechnological characteristics.

### *Bacillus* sp. G51 extracellular proteolytic activity

The optimal temperature for proteolytic activity of *Bacillus* sp. G51 was approximately 60 °C (Fig. 2a). It was stable at moderate temperatures, showing about 90% residual activity after 2 h of incubation at 40 °C (Fig. 2b). The residual proteolytic activity decreased to approximately 30% after 2 h at 50 °C, and no residual activity was detected after 2 h at 60 °C (Fig. 2b). Moreover, proteolytic activity was highly stable in the pH range of 6.0–11.0, with an optimum for activity at pH 9.0 (Fig. 2c, d). Feather-degrading serine proteases from *Bacillus mojavensis* A21 showed optimum pH for proteolytic activity between 8.0 and 11.0 [30]. The optimum pH of proteases produced by wool-degrading *Bacillus amyloliquefaciens* MA20 and *B. subtilis* MA21 was 9.0 [31]. A positive characteristic of G51 proteolytic activity was its high stability to non-ionic surfactants (Triton X-100 and Tween 20) and to surfactants used in wool processing (Lipocol OXO 650 and Teridol B), (Table 1). In contrast, residual activity decreased to approximately 40% with the anionic surfactant SDS (Table 1). Even though $Ca^{2+}$ may provide structural and thermal stability to some proteases, it only marginally enhanced the proteolytic activity of G51 (Table 1). $Zn^{2+}$ reduced G51 proteolytic activity (Table 1); this could be related with inhibition of metalloproteases due to the additional binding of $Zn^{2+}$ (in the mM range) to the binding site [32]. In addition, the high stability of G51 proteolytic activity to $H_2O_2$ is an important feature (Table 1), as this is used as a bleaching agent and in wool shrink-resist pre-treatments [11, 12, 33].

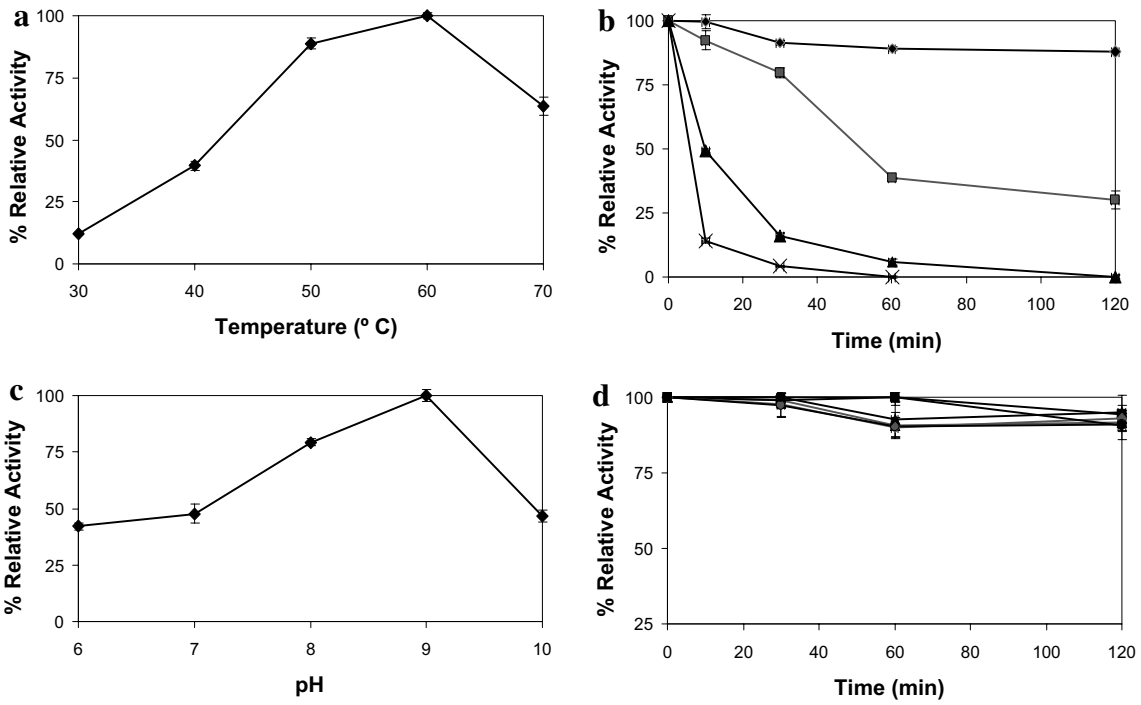


**Fig. 2** Effects of temperature and pH on the activity and stability of *Bacillus* sp. G51 proteolytic activity. **a** Determination of optimal temperature for activity. **b** Determination of thermal stability; cell-free supernatant was incubated at 40 (*filled diamond*), 50 (*filled square*), 60 (*filled triangle*) and 70 °C (*cross*) without substrate, and then the residual activity was measured. **c** Determination of optimal pH for activity. **d** pH stability experiments; enzyme extract was incubated in buffers with the following pH values 6 (*open triangle*), 7 (*filled square*). 8 (*filled triangle*), 9 (*filled diamond*), 10 (*filled circle*) and 11 (*open circle*), and then the residual activity was determined. The 100% value for the proteolytic activity corresponded to 1.5 CAU/mL. Data: means ($n = 3$) ± 1 SE

**Table 1** Effect of protease inhibitors and chemical agents on *Bacillus* sp. G51 proteolytic activity

| Chemical agents | Concentration | Residual activity (%) |
|---|---|---|
| E64 | 0.1 mM | 95.05 ± 1.06 |
| Iodoacetamide | 0.1 mM | 103.00 ± 5.46 |
| PMSF | 1 mM | 41.32 ± 1.09 |
| PMSF | 10 mM | 34.50 ± 3.18 |
| EDTA | 10 mM | 27.68 ± 0.73 |
| 1,10-Phenanthroline | 10 mM | 37.69 ± 1.02 |
| Triton X-100 | 1% v/v | 102.97 ± 3.88 |
| Triton X-100 | 5% v/v | 80.52 ± 2.07 |
| Tween 20 | 1% v/v | 111.31 ± 3.04 |
| Tween 20 | 5% v/v | 121.97 ± 3.73 |
| SDS | 1% w/v | 38.98 ± 1.69 |
| Lipocol OXO 650 | 1% v/v | 99.86 ± 2.19 |
| Teridol B | 1% v/v | 98.36 ± 6.00 |
| β-Mercaptoethanol | 5 mM | 83.92 ± 8.40 |
| DTT | 5 mM | 71.63 ± 4.33 |
| $H_2O_2$ | 1% | 97.01 ± 0.29 |
| $ZnSO_4$ | 5 mM | 79.00 ± 0.54 |
| $CaCl_2$ | 5 mM | 103.90 ± 0.97 |

Data: means ($n = 3$) ± 1 SE

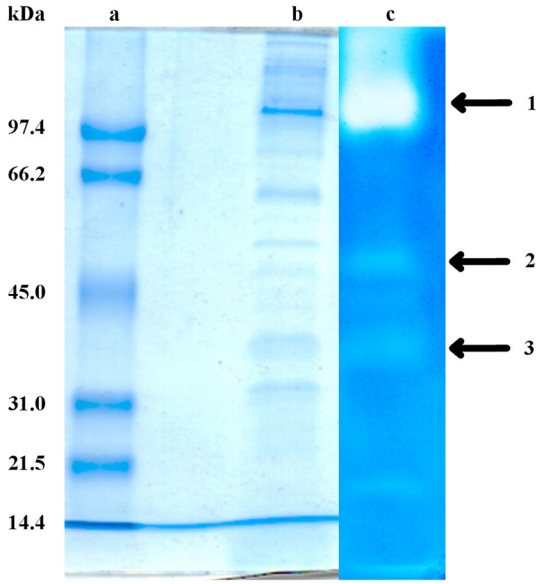


**Fig. 3** Non-reducing SDS–PAGE and zymogram analysis from culture supernatant of *Bacillus* sp. G51. *Line a* Low range molecular mass markers (Bio-Rad). *Line b* protein profile of culture supernatant of *Bacillus* sp. G51. *Line c* culture supernatant of *Bacillus* sp. G51 zymogram. *1, 2*, and *3* bands with proteolytic activity

G51 proteolytic activity was not inhibited by the inhibitors for cysteine proteases E-64 and iodoacetamide (Table 1). In contrast, it was partially inhibited by the serine protease inhibitor PMSF and the metalloprotease inhibitors EDTA and 1,10-phenanthroline (Table 1), suggesting a mixture of serine and metalloproteases in G51 culture supernatant. Non-reducing SDS–PAGE and zymogram showed a protein band with the highest proteolytic activity and the lowest electrophoretic mobility (Fig. 3, band 1) and other bands with lower activity and different mobility (Fig. 3, bands 2 and 3). LC-MS/MS analysis of released tryptic peptides of those bands resulted in the identification of peptides belonging to different proteases (Table 2). Proteolytic band 1 was associated with Subtilisin E (Table 2; Fig. 3), an extracellular enzyme with $Ca^{2+}$ ions as cofactor. Proteolytic bands 2 and 3 delivered peptides matching with the metalloprotease Bacillolysin and with an intracellular serine protease, respectively (Table 2; Fig. 3). The serine proteases belong to the peptidase family S8 of MEROPS database that contains Subtilisin and its homologues. Subtilisin-like serine proteases show a preference for cleavage after hydrophobic residues or dibasic amino acids, and are important in the degradation of keratinaceous substrates [34, 35]. Other studies demonstrated that Subtilisins with high specificity for aromatic and hydrophobic amino acids in the P1 substrate position efficiently degraded feather keratin, possibly due to the number of residues either aromatic or hydrophobic (approximately 50%) found in keratin molecules [36, 37]. Bacillolysin (family M4) is an extracellular $Zn^{2+}$ dependent enzyme with four $Ca^{2+}$ ions as cofactor. Most members of family M4 degraded proteins and peptides with a preference for cleavage of Xaa + Yaa, in which Xaa is a hydrophobic residue and Yaa is Leu, Phe, Ile, or Val (http://merops.sanger.ac.uk).

Interestingly, protein bands 2 and 3 also delivered peptides matching with dihydrolipoyl dehydrogenases and γ-glutamyltranspeptidase (GTT) which could be involved in keratin degradation (Table 2). In addition to hydrogen bonds and hydrophobic interactions, keratin is strongly stabilized by cross-linking of protein chains through disulfide bonds [35]. The secondary structural conformation of wool keratin corresponds to hard α-keratin which is characterized by a high level of disulfide bonds, and hence high recalcitrance to proteolytic degradation [38]. Sulfitolysis, the breakdown of disulfide bonds, might be accomplished by disulfide reductases, chemical or live cell redox [39]. Previous findings suggested that keratin degradation is a cooperative process which includes keratinases and disulfide reductases [40–42]. Dihydrolipoyl dehydrogenases are disulfide oxidoreductases that catalyze the oxidation of dihydrolipoamide to lipoamide. GTT is one of the enzymes (aminoacyltransferase/hydrolase) implicated in feather keratin degradation by *B. subtilis* CH-1 [40]. A monomeric GGT30 from *Bacillus licheniformis* ER-15, produced by proteolytic digestion of a GGT67 by Subtilisin, assists Subtilisin during cell free degradation of α-keratin of hooves and nails

**Table 2** Proteins identified by LC-MS/MS analysis

| Band | Accession code | Sequence description | Number of unique peptides | Enzyme description |
|---|---|---|---|---|
| 1 | P04189 | Subtilisin E | 2 | Extracellular alkaline serine protease (EC 3.4.21.62). Family S8 |
| 2 | P68736 | Bacillolysin | 2 | Extracellular zinc metalloprotease (EC 3.4.24.28). Family M4 |
| | P21880 | Dihydrolipoyl dehydrogenase | 5 | Oxidoreductase (EC 1.8.1.4). Class-I pyridine nucleotide-disulfide oxidoreductase family |
| | P54533 | Dihydrolipoyl dehydrogenase | 3 | Oxidoreductase (EC 1.8.1.4) |
| | P54422 | γ-glutamyl transpeptidase | 1 | γ-glutamyltransferase (EC 2.3.2.2). |
| 3 | P11018 | Major intracellular serine protease | 2 | Serine endopeptidase (EC 3.4.21). Family S8 |
| | P54422 | γ-glutamyl transpeptidase | 4 | γ-glutamyltransferase (EC 2.3.2.2) |

[43, 44]. Sharma and Gupta [42] demonstrated that γ-glutamyltranspeptidase (GGT)-glutathione (GSH) system is involved in the degradation of feathers by reducing disulfide bonds making them more vulnerable to keratinase attack.

Enzyme purification is an important factor for the industrial application of enzymes due to its impact on the production costs. In addition, purification steps may remove important components responsible for the reduction of keratin disulfide bonds [38, 41]. G51 crude enzyme extract has the advantage of containing a combination of different peptidases in addition to other enzymes that could be involved in the reduction of disulfide bonds (e.g. GTT) that may cooperate in the degradation of the wool cuticle, inducing the descaling of the fibers (Online Resource 2). In line with these results, Chaya et al. [45] found that only the crude enzymes secreted by *Fusarium oxysporum* 26-1 or the combination of two of them, rKrtA (S8 family peptidase) and rKrtC (M36 family peptidase), significantly descaled wool fibers. Congruently, *Stenotrophomonas maltophilia* BBE11-1 produced two keratinases and one protein with the ability to break disulfide bonds that degraded wool and feather in a synergic manner [46]. Moreover, G51 proteolytic activity showed high stability to β-mercaptoethanol and DTT (Table 1) which is another positive feature because reducing agents such as sodium sulphite, β-mercaptoethanol, cysteine, and DTT have been applied to enhance keratin sulfitolysis [37].

## Wool felt-resist treatment using G51 proteases

Both *Bacillus* sp. G51 proteases and Esperase significantly increased the felt-ball diameter (1.1 and 1.2 mm, respectively), and consequently decreased the felt-ball density, in comparison to those of untreated wool (Table 3; Fig. 4). It has been suggested that felt-ball diameter would only have to increase approximately to 30–32 mm to meet the machine wash specifications for the shrink-proofing felt-ball test [47]. Allam et al. [48] reached 30.2 mm felt-ball diameter (40.4 μm wool fiber) by optimizing a keratinase treatment. In this study, the wool weight loss was low (<1.5%) for all treatments. Particularly, the weight loss of wool treated with *Bacillus* sp. G51 proteases did not differ significantly from that of the control treatment (Table 3). This low weight loss of wool could be related with the moderate incubation conditions selected for the treatment with G51 proteases far from the optimal pH and temperature conditions (Fig. 2). On the other hand, wool treatment with Esperase resulted in a significantly higher weight loss than in controls (Table 3). In line with these results, partial descaling of the wool fibers was observed after wool treatment with G51 proteases, while regions with excessive enzymatic attack to the fiber were detected in Esperase treated wool (Online Resource 2). After wool treatment with native proteases from *Bacillus lentus* and *B. subtilis*, Jus et al. [49] reported a similar increase in felt-ball diameters to that found in this study but with higher weight losses (4.79 and 14.67%, respectively, after 180 min incubation).

**Table 3** Weight loss, felt-ball diameter and density of wool top treated with *Bacillus* sp. G51 proteases, Esperase (SIGMA), and control without enzymes

| Treatment | Felt-ball diameter (cm) | Felt-ball density (g/cm$^3$) | Weight loss (%) |
|---|---|---|---|
| Esperase | 3.00 ± 0.02 b | 0.068 ± 0.001 a | 1.09 ± 0.14 b |
| G51 proteases | 2.99 ± 0.03 b | 0.071 ± 0.003 a | 0.31 ± 0.08 a |
| Control without enzymes | 2.88 ± 0.01 a | 0.079 ± 0.001 b | 0.38 ± 0.06 a |

Data: means ($n = 5$) ± 1 SE. Lowercase letters: significant differences among treatments ($p < 0.05$)

**Fig. 4** Wool felt-balls after treatments (from *left* to *right*): control without enzymes, *Bacillus* sp. G51 proteases, and Esperase

High weight loss values have been associated with the diffusion of proteases inside the wool fiber causing undesirable hydrolysis and strength loss [2]. It was reported that commercial processes aim for a 3–6% weight loss after enzymatic hydrolysis [50].

## Conclusion

Enzymatic treatments could be an environmentally friendly alternative for reducing top felting. Patagonian Merino wool harbor bacteria from different genera which secreted wool-degrading enzymes. *Bacillus* sp. G51 produced a combination of extracellular serine and metallopeptidases which caused a decrease in felting tendency of wool top without significant weight loss. This treatment based on peptidases from a wild bacterium has potential for meeting the demands of organic wool processing which bans the use of hazardous chemicals and genetic engineering.

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**Compliance with ethical standards**

**Conflict of interest** No conflict of interest declared.

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