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Identification of a Keratinase-Producing Bacterial Strain and Enzymatic Study for Its Improvement on Shrink Resistance and Tensile Strength of Wool- and Polyester-Blended Fabric

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Abstract A wool-degrading bacterium was isolated from decomposition wool fabrics in China. The strain, named 3096-4, showed excellent capability of removing cuticle layer of wool fibers, as demonstrated by removing cuticle layer completely within 48 h. According to the phenotypic characteristics and 16S rRNA profile, the isolate was classified as *Pseudomonas*. Bacteria growth and keratinase activity of the isolate were determined during cultivation on raw wool at different temperatures, initial pH, and rotation speed using orthogonal matrix method. Maximum growth and keratinase activity of the bacterium were observed under the condition including 30 °C, initial pH 7.6, and rotational speeds 160 rpm. The keratinase-containing crude enzyme prepared from 3096-4 was evaluated in the treatment of wool fabrics. The optimal condition of our enzymatic improvement of shrink resistance was the combination of 30 °C, initial pH 7.6, and rotation speeds 160 rpm. After the optimized treatment, the wool fabrics felting shrink was 4.1% at 6 h, and textile strength was not lost.

Keywords *Pseudomonas* · Keratinase · Wool fabrics · Shrink resistance

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Introduction

Wool is a multifunctional fiber with a range of diameters that make it suitable for clothing, household fabrics, and technical textiles. Its ability to absorb and release moisture makes woolen garments comfortable as well as warm. Two thirds of wool is used in the manufacture of garments, including sweaters, dresses, coats, suits, and active sportswear. Although wool is welcomed all over the world as a kind of high-grade textile materials, the market value of wool is limited by the fact that consumers place increasingly high demands on machine washability and softness. Felting shrinkage is a typical property of wool due to the configuration of the scales of the wool fiber, especially during washing.

Chlorine-Hercosett and enzyme processes currently represent the main methods to control wool felting shrinkage. Chlorine-Hercosett process, an effective commercial shrink-resist finishing, has been used for decades. One of the major concerns for this process is the environmental pollution problem caused by the disposal of absorbable organic chlorides (AOX). Enzyme process is, however, an environmentally friendly process, in which especially proteases and lipases have been utilized for the modification of wool fiber [1], whereas current enzyme processes are difficult to control and are not sufficiently predictable and reproducible in industrial scale due to the chemical pretreatment. Such treatment can cause excessive damage to the fiber cuticle with consequent high levels of weight and tensile strength loss [2, 3].

Hence, there is an urgent need for the development of environmentally friendly and controllable processes for wool shrink-resist finishing. The felting shrinkage and unsoftness of wool fabrics were attributed to scale layers. Scale layers are the outer layer of wool fiber, affecting the dying property of wool. So removing scale layers or decreasing effect of scale layers is the main process of wool finishing. Scale layers constitute keratin that contains rich disulfide bonds and resists degradation by common proteases. In the nature, however, wool never has been accumulated, largely attributing to degradation by keratinase secreted by several microbes. Therefore, keratinase has been considered to be a promising tool for removal of wool scale layers and has been investigated by different labs [4, 5]. In contrast, the capability of keratinase in improving wool fabrics' shrinkage resistance was less understood.

The aim of this study was to find a new bacterial isolate that possesses the ability to degrade wool scale layers at lower temperature. The enzyme produced by this bacterial strain may decrease wool felting shrinkage through only one step and be potentially applied to wool textile finishing process.

Materials and Methods

Isolation of Wool Keratin-Degrading Bacterium

Decomposed wools were collected from several sites at a local wool textile plant. Wools were flooded in peptone broth (5 g/L) and incubated at 37 °C for 24 h. The suspension was used to streak wool meal agar plates (wool meal 20 g/L, NaCl 5 g/L, K_2HPO_4 3 g/L, KH_2PO_4 4 g/L, and agar 20 g/L, pH 7.4–7.6), which were incubated at 37 °C for 3 days. Single colony was isolated and screened for their ability to hydrolyze keratin in wool

powder agar plates. Colonies producing clear zones in this medium were selected for further analysis.

Molecular Phylogenetic Studies

Genomic DNA was extracted as follows. Briefly, the strain, which had the highest enzyme activity, was inoculated into beef extract/peptone/NaCl medium and incubated with shaking at 30 °C for 16 h. Cultures (1.5 mL) were centrifuged at 7,000 rpm for 10 min, supernatant was removed, and the pellet was resuspended in 10 μL lysozyme (50 mg/mL) and 0.5 mL sterilized deionized water, followed by mixing with 500 μL DNA extraction buffer (4% SDS 100 mM Tris–HCl, 10 mM EDTA, pH 8.0) at 65 °C for 1 h. The suspension was extracted by mixture of phenol and chloroforms (1:1 volume) and then was extracted by pure chloroform again. DNA was precipitated by the adding two volumes of pre-cooling 100% ethanol. The pellet was then dissolved into 50 μL distilled water and quantified by spectrophotometer [6].

The 16S rRNA gene was amplified by the polymerase chain reaction (PCR) with the primers AGA GTT TGA TCC TGG CTC AG (position 8–27), AAG GAG GTG ATC CAG CCG CA(position 1,541–1,522) (http://www.psb.ugent.be/rRNA/). The PCR reaction mixtures (50 μL) each contained dNTPs 200 μmol/L, 10× PCR buffer 5 μL, 1.5 mM $MgCl_2$, 50 pmol each primers, *Taq* DNA polymerase 1.5 U, and genomic DNA 0.1–2 μg. The PCR was carried out with initial denaturation of 94 °C for 5 min followed by 32 cycles of denaturation at 94 °C for 1 min, annealing at 53 °C for 30 s, extension at 72 °C for 90 s, and a final extension at 72 °C for 10 min. Amplified DNA fragments were detected by horizontal electrophoresis in 1.0% agarose gel containing ethidium bromide (0.5 μg/mL) at 100 V for 0.5 h in 1× TAE buffer with 3 μL aliquots of PCR products. PCR products were sent to Shanghai Sangon Biological Engineering Technology & Services Co, Ltd (Shanghai, China) for sequencing.

The BLAST algorithm was used to search for homologous sequences in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Molecular phylogenetic studies of the 16S rRNA were conducted using MEGA 4.0 [7].

Bacteria Growth and Enzyme-Produced Conditions

To find the optimal culture requirements, the following factors were investigated using the orthogonal matrix method, each constituent of temperature, initial pH value, and rotation rate. The isolate from a 10^6 colony forming units per milliliter culture was cultivated in LB medium solution (bacto-tryptone 10 g, bacto-yeast extract 5 g, NaCl 10 g, dH_2O to 1 L). The medium (100 mL) containing 10 mL isolate was added into 500 mL flasks for shaking culture. The bacterial culture was cultured for 16 h and diluted to 10× or 20× by deionized water for bacterial growth measurement (600 nm).

The enzyme activity was assayed by the following procedures [6, 8]. The reaction mixture contains 1 mL of crude enzyme and 2 mL of 10 mg keratin powder in 0.05 mol/L Tris–HCl (pH 7.6) buffer. The mixture was incubated at 30 °C for 1 h, and the enzyme reaction was stopped by adding 2 mL of 10% trichloroacetic acid. After centrifugation at 10,000 rpm at 4 °C for 10 min, the absorbance of supernatant was determined at 280 nm. One unit of enzyme activity corresponds to the amount of enzyme that causes a change of absorbance of 0.1 at 280 nm at 30 °C for 1 h.

All data were obtained before by variance analysis. Differences of $F>1$ were considered statistically significant levels.

Treatment of Wool Fabrics with Enzymes

Worsted wool fabrics (unbleached 80% wool yarn and 20% polyester yarn, made from 64 s wool fiber, 21–23 μm) were bought from Shanghai Greenmei Textile Corp., China and used throughout the study.

Based on the orthogonal experiment, optimum conditions (including culture temperature, initial pH, and rotational speeds) were selected and applied in following experiments of bacterial growth and enzymes production. The bacteria were cultivated in 200 mL of medium in a 500-mL triangular flask and grown at initial pH 7.6, 30 °C, and 160 rpm for 48 h. Then, the culture was treated by ultrasonication on ice at 150 W for 3 min. The crude extraction was prepared by centrifugation at 4 °C and 10,000×*g* for 10 min. Protein in the liquid was precipitated by adding ammonium sulfate with gentle stirring until the concentration of ammonium sulfate reached 30%, and then left to stand for 2 h and centrifuged at 4 °C, 10,000×*g* for 10 min. The precipitate was dissolved to 2 U/mL in a buffer solution (0.05 mol/L Tris–HCl, pH 7.6).

In the assays of enzyme treatment for wool fabrics, initial pH ranging from 7.2 to 8.0 in 0.2 increments at 30 °C and temperature in the range of 20, 24, 28, 30, 32, and 36 °C at initial pH 7.6 was investigated. Wool fabrics (100×100 mm samples) were incubated with 2 U/mL enzymes. The reaction was allowed to proceed for 12 h on the shaking table, and the rotational speed was 300 rpm. Control samples, without enzymes and treated only with buffer solution, were checked as well, following exactly the same treatment condition as aforementioned.

Analytical Methods

Washing Tests to Assess Wool Fabric Shrinkage and Decrement Rate

After treatment as aforementioned, wash area shrinkage tests were conducted at 40 °C by using a standard detergent with laundry powder 4 g/L, neutral soap flakes 0.5 g/L, bath ratio 1 : 20, wool fabric 0.8 kg to wash for three times, and further washing with clear water for two times. Dewatering and drying were then conducted to reach the balance of moisture absorption and release. Felting shrinkage were calculated using the formula below:

Felting shrinkage (%)=(1−wool fabrics' size after washing/wool fabrics' size before washing)×100

Wool fabrics were dried to constant weight at 105 °C before and after treatment by enzymes, and calculated decrement rate used the formula below:

Percentage of weight loss (%)=(1−wool fabrics weight after enzymes' treatment/wool fabrics weight before enzymes' treatment)×100

Tensile Strength of Fabrics

Tensile strength of fabrics was evaluated by material testing machine (H10K-S, Tiniius Olsen Inc., USA). Samples were balanced at 25 °C with a relative humidity of 60% for 24 h before testing. Each sample was in dimension of 50×50 mm after drawing out the yarns of 5 mm at both sides. Tensile strength of wool fabrics was only tested in the warp direction.

Morphological Study on Wool Fabrics Using SEM

The morphology of the treated and untreated wool fabrics was observed using a scanning electron microscope (SEM JSM-5600LV, Jeol, Japan). The samples were coated with gold before SEM testing.

Results

Isolation of Wool Keratin-Degrading Bacterium

Distinct colony morphologies were observed on wool agar plates. These isolates were characterized for the purpose of their keratin-degrading properties. They were cultured on wool meal agar plates and transferred to wool meal broth plates for monitoring the activity of keratin degradation. The isolate, 3096-4, showed the highest keratinase activity among these isolates. Raw wool fibers were removed, and the fiber was damaged in different degree by 3096-4 after 48 to 72 h at 30 °C (Fig. 1). As shown in Fig. 1, the wool fibers' cuticle layers were removed completely after 48 h, but wool fiber revealed significant proteolytic damage after 72 h.

Further characterization for strain 3096-4 was performed by molecular phylogenetic analysis and morphology observation. Based on 16S rRNA sequencing data, the taxonomic properties of strain 3096-4 were summarized in Table 1 and Fig. 2. The 16S rRNA sequence of strain 3096-4 (GU083600) showed high similarity to a group consisting of several *Pseudomonas* strains, for example, sharing a 99% similarity with the *Pseudomonas aeruginosa* strain ZFJ-1 (EU931548). Bootstrap analysis also resulted in relatively high values for the branching of 3096-4 within the *Pseudomonas* cluster. Other related members of the *Erwinia*, *Shigella*, and *Escherichia* shared 95–98% sequence similarity with strain 3096-4 (Fig. 2). These results indicated that the isolate phylogenetically belongs to *Pseudomonas*. Cellular/colony morphology and growth characteristics of the isolate were also more resembling to those of *Pseudomonas* (see Table 1 and Fig. 2). We designated this new isolate as *Pseudomonas* sp. strain 3096-4.

Optimal Bacteria Growth Conditions

Culture condition is important for bacterial growth and enzyme production. According to the single factor experiment, which was used for isolation of the isolate 3096-4 (data not shown), the culture conditions including temperature, initial pH, and rotational rate were further optimized using an orthogonal layout L9 (3^3). Factors, levels design, and the experimental results of orthogonal layout are detailed in Table 2. As shown in the table, the fastest bacterial growth of the isolate was corresponding to 0.75 (OD) which was found in the fifth group among total nine experimental groups, with the growing factors of 30 °C, initial pH 7.6, and 200 rpm rotational rate.

Analysis of culture condition and keratinase production by isolate 3096-4 in shake flask culture with L6 (3^3) orthogonal test are shown in Table 3. Optimal level of bacterial growth and enzyme production included temperature (30 °C), initial pH (7.6), and rotational rate (160 rpm). Based on the magnitude order of R value (maximum difference), the order of effects of all factors on bacterial growth and enzyme production was temperature>rational speed>initial pH, and temperature is the most important factor.

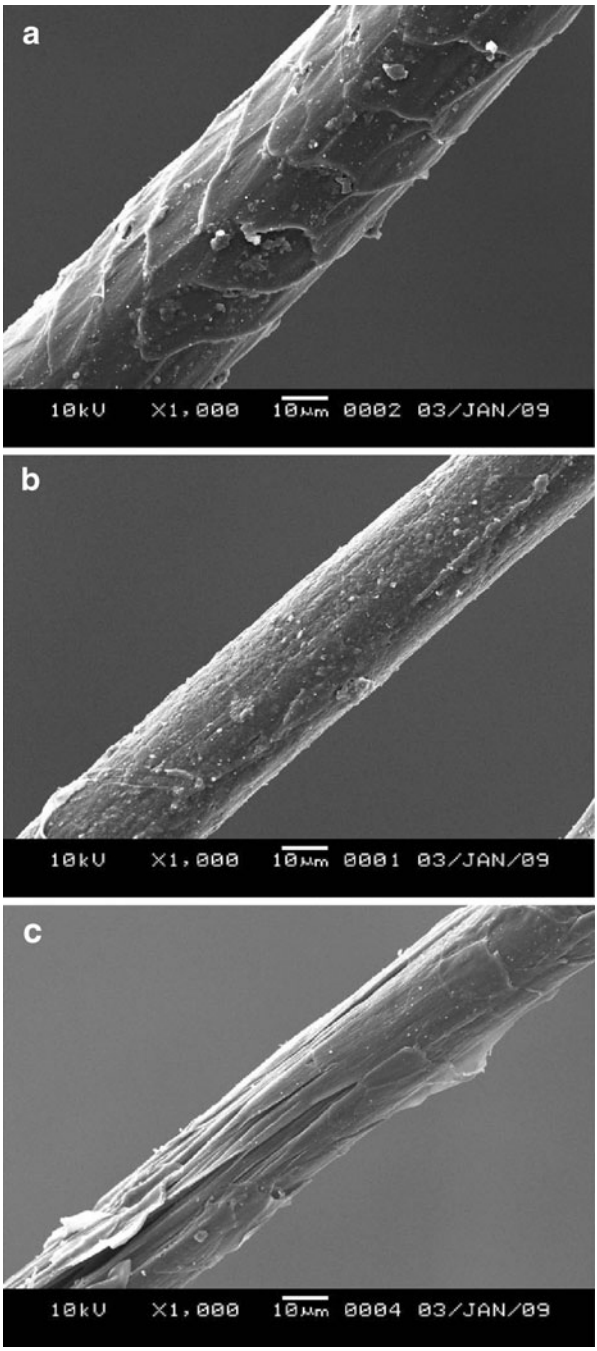

Fig. 1 Transmission electron micrograph of wool. **a** Control. **b** Treatment by isolate 3096-4 for 48 h. **c** Treatment by isolate 3096-4 for 72 h

Treatment of Wool Fabrics with the Crude Enzymes

The wool fabrics' properties, including the felting shrinkage, tensile strength, and percentage of weight loss, were measured after keratinase treatment at different conditions

Table 1 Morphological characteristics of keratinase-producing bacterium 3096-4.

Morphological characteristics				Cultural characteristics
Form	Size (μm)	Gram stain	Spore	
Rods	0.3–0.6×0.8–1.5	Negative	Non-sporulating	Wool meal agar colonies, red color, circular, smooth, convex, moist

without any pretreatment (Fig. 3). As shown in Fig. 3a, the felting shrinkage value of wool fabrics decreased to 2.5% at 30 °C and then increased with the elevated temperature. Fig. 4a shows that the wool fabrics' felting shrinkage reached the lowest value of 2.7% at pH 7.6 but then increased with higher pH condition.

Interestingly, although the weight loss of wool fabrics after keratinase treatment was remarkably high in these experiments as shown in Fig. 3c and Fig. 4c, the tensile strength of wool fabrics was reversely correlated with the decrease of felting shrinkage according to the data in Fig. 3b and Fig. 4b. It was probably due to the permeation of proteins and amino acids derived from keratin-degraded wool fibers, which enhanced the fiber strength.

To optimize the enzyme treating time for wool fabrics, which is a key factor in industrial applications, the wool fabrics were treated for different time periods, 6 and 15 h under the optimum temperature and initial pH condition. The felting shrinkage, tensile strength, and percentage of weight loss were subsequently assayed. As shown in Fig. 5, the felting shrinkage was 4.1%, and the tensile strength remained undetectably changed when treatment time is less than 6 h, whereas during the prolonged treating time, the felting shrinkage was decreased, and tensile strength was increased.

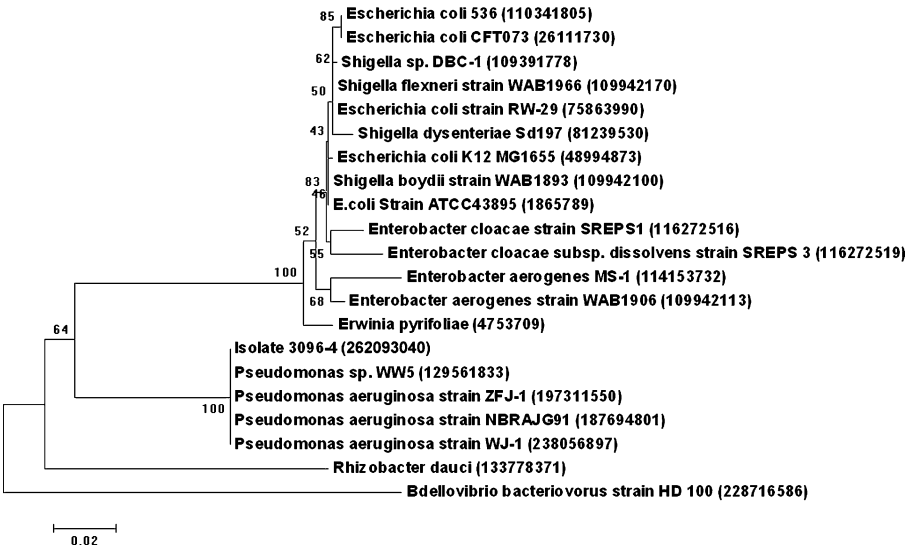

Fig. 2 Phylogenetic tree of 16S rRNA of strain 3096-4 within the genus *Pseudomonas* and allied bacteria. The branching pattern was generated by the neighbor-joining method. *Bar* Jukes-Cantor distance of 0.005. *Bdellovibrio bacteriovorus* was used as an outgroup. *GenBank register numbers* in the *brackets*

Table 2 Results and variance analysis of L6 (3^3) orthogonal test of the isolate 3094-4 and enzyme product in shake flask culture.

Exp. group	Temperature (°C)	Initial pH	Rotational speed (rpm)	Bacterial growth (OD)[a]	Enzyme activity (U)[a]
1	25	7.2	120	0.31	1.33
2	25	7.6	160	0.42	1.83
3	25	8.0	200	0.37	1.71
4	30	7.2	160	0.70	3.21
5	30	7.6	200	0.75	3.11
6	30	8.0	120	0.63	3.37
7	35	7.2	200	0.51	2.94
8	35	7.6	120	0.48	2.16
9	35	8.0	160	0.55	2.76

[a] Values are mean of triple determinations

To determine surface change of wool fabrics, we analyzed the SEM images of the keratinase-treated fabrics (Fig. 6). As shown in Fig. 6a, slight cuticle layers of the fibers were removed, and there were many fragments on the surface of the wool. These fragments come from mechanical damage of textile process. The fragments were removed by keratinase treatment after 6 h, and the wool fibers were transformed into smooth surface (Fig. 6b). With the increased treatment time, such as more than 15 h, the surface of wool fabrics produced some fragments again (Fig. 6c). This was caused by degraded of partial cuticle layer.

Table 3 Analysis of culture condition and keratinase production by isolate 3096-4 in shake flask culture with L6 (3^3) orthogonal test.

	Bacterial growth (OD)			Enzyme activity (U)		
	Temperature (°C)	Initial pH	Rotational speed (rpm)	Temperature (°C)	Initial pH	Rotational speed (rpm)
K1[a]	0.367	0.507	0.473	1.623	2.493	2.287
K2	0.693	0.550	0.557	3.230	2.367	2.600
K3	0.513	0.517	0.543	2.620	2.613	2.587
Range	0.326	0.043	0.084	1.607	0.246	0.313
Optimal level	30	7.6	160	30	7.6	160
Sum of squares (SS)	0.161	0.003	0.012	3.947	0.091	0.188
Degree of freedom (f)	2	2	2	2	2	2
F	2.744	0.051	0.205	1.955	0.045	0.093
<rho> values	0.026	0.0005	0.002	0.658	0.015	0.031

[a] K1=the mean of bacterial growth in thrice experiment at Xi

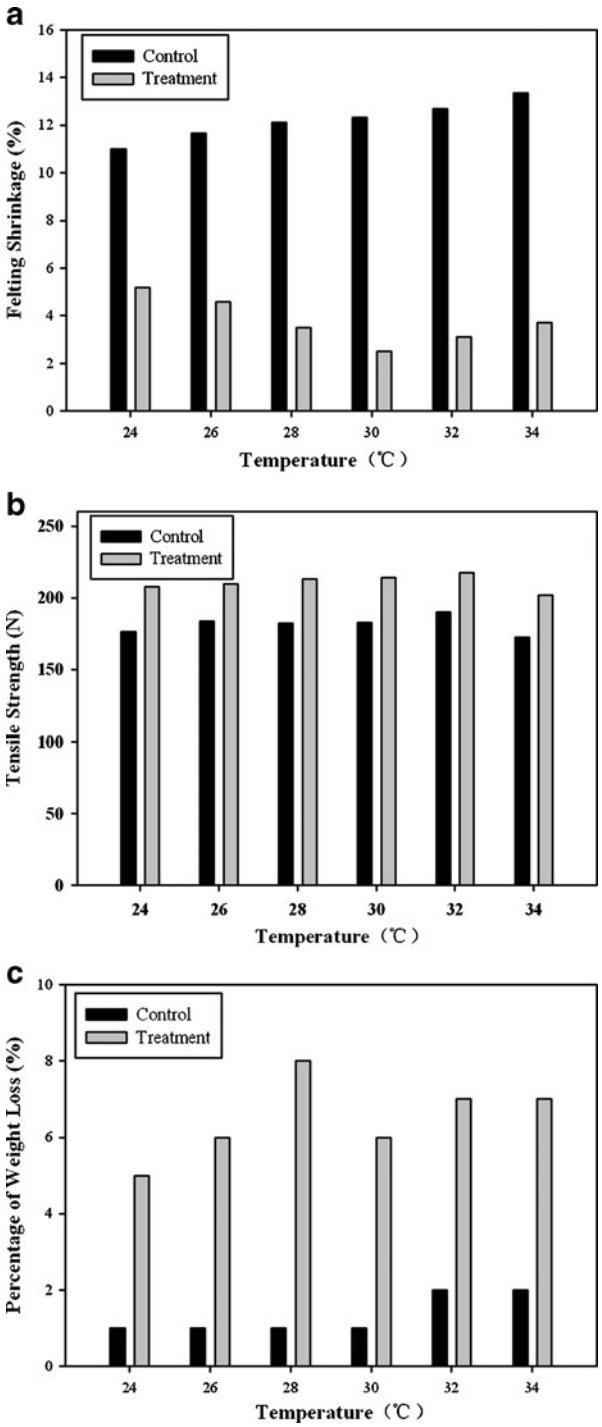

Fig. 3 Effect of temperature on felting shrinkage (**a**), tensile strength (**b**), and percentage of weight loss (**c**) of wool fabrics after treatment of crude keratinase from isolate 3096-4. Control is the treatment with Tris–HCl (pH 7.6)

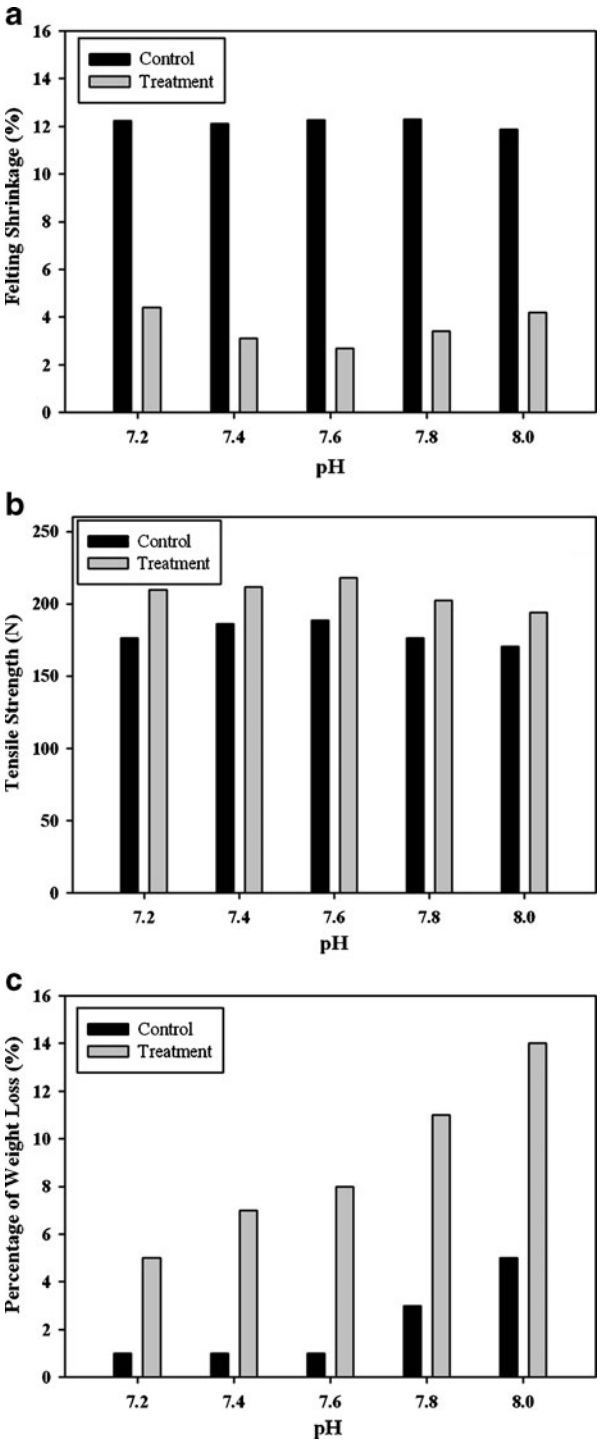

Fig. 4 Effect of pH on felting shrinkage (**a**), tensile strength (**b**), and percentage of weight loss (**c**) of wool fabrics after treatment of crude keratinase from isolate 3096-4. Control is the treatment with Tris–HCl (pH 7.6)

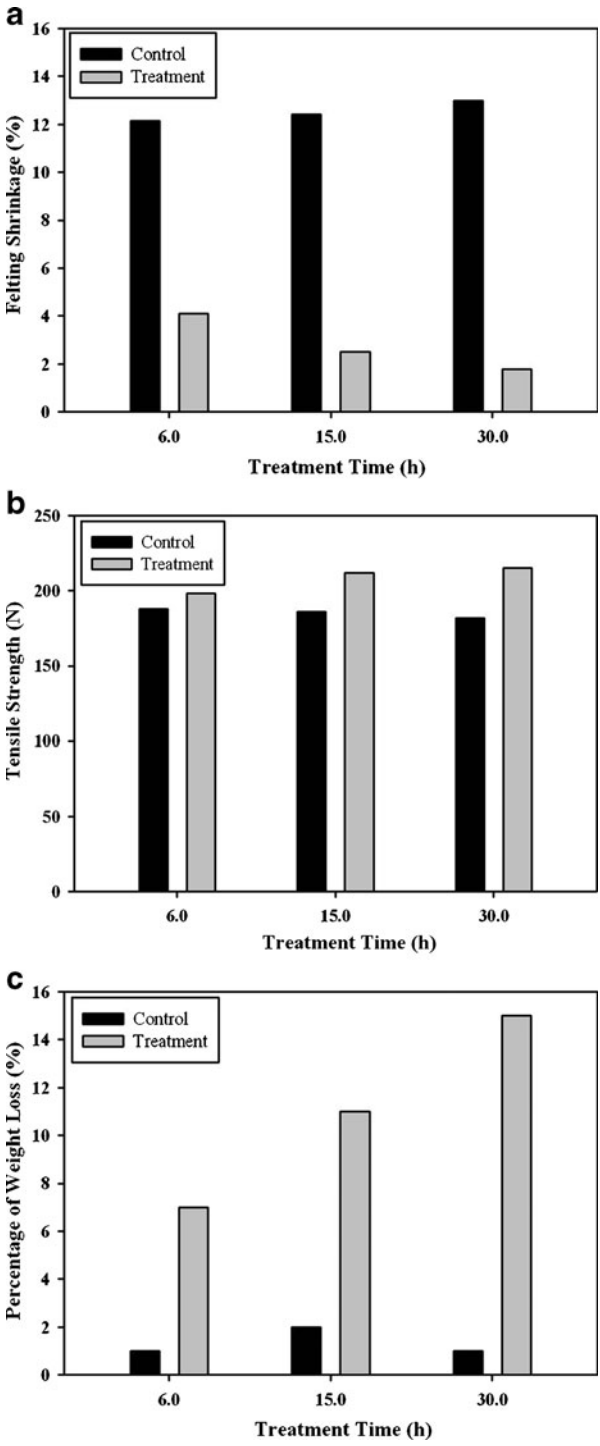

Fig. 5 Effect of different treatment time on felting shrinkage (**a**), tensile strength (**b**), and percentage of weight loss (**c**) of wool fabrics after treatment of crude keratinase from isolate 3096-4. Control is the treatment with Tris–HCl (pH 7.6)

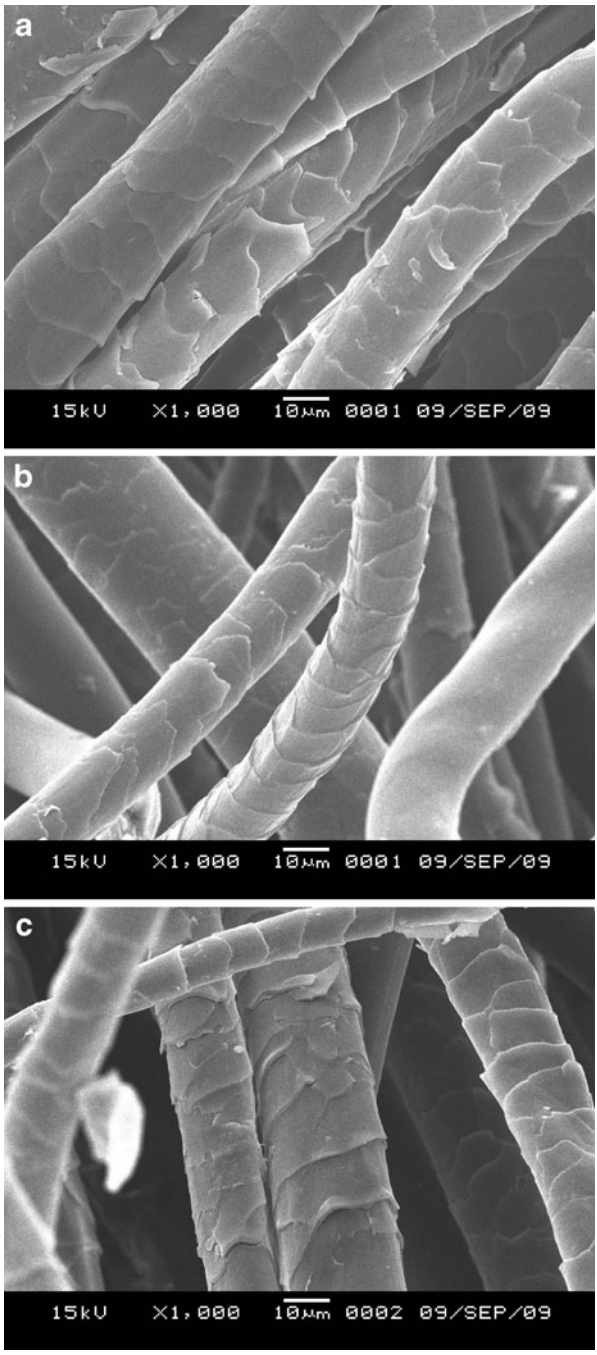

Fig. 6 Transmission electron micrograph of wool fabrics. **a** Control. **b** Treatment by isolate 3096-4 for 6 h. **c** Treatment by isolate 3096-4 for 15 h

Discussion

Chlorine-Hercosett, the most mature and general application process in the industry, had been published in many textbooks. Felting shrinkage of wool fabrics is 2.5% within 20 min

chlorine (dichloro-isocyanuric acid) treatment [9], but the decrease of fabrics tensile strength is violent. Therefore, an addition process, polymer treatment, is necessary, which increased treatment step and cost. More important, Chlorine-Hercosett process could cause the disposal of absorbable organic chlorides (AOX), and it is an environmental pollution problem. When environmental considerations are becoming more important in the textile finishing industry, traditional chemical anti-felting treatments are subject to increasing criticism [1]. Enzyme processes, a hallmark of green textile, were the most ideal method in textile industry, but disulfide bonds are the most abundant cross links in wool fibers, and they are the main bonds responsible for the stabilization of wool fibers. Therefore, most of proteases cannot break it down. In the protease-based process of anti-felting treatment for wool fabrics, pretreatment was indispensable. Currently, the general process of pretreatment comprises chemical oxidation, peroxidase, catalase (or lipase) treatment, and protease treatment [10]. Such treatment process, besides removal of the cuticle layer, can cause excessive proteolytic damage to the fiber with consequent high levels of weight and tensile strength loss due to penetration of the protease into the bulk of the fibers. So, three approaches have been proposed to overcome this limitation in this kind of protease processing of wool fabric treatment. The first one was resin treatment, which increased wool fibers' tensile strength. The second was to limit the action of the proteases only to the fibers' surface by increasing their molecular size through grafting soluble polymers adducts, e.g., PEG on the proteins [11]. The third approach was to apply transglutaminase (EC2.3.2.13) to remediate the negative effects of protease treatments in terms of loss in fiber strength [3, 10]. Transglutaminase is an enzyme capable of catalyzing acyl transfer reactions by introducing covalent crosslinks among proteins as well as peptides and various primary amines. These processes increased the treatment steps and cost, so the application of proteases for anti-shrink wool could therefore not find widely industrial application [2, 3].

Keratinase is capable of degrading cuticle layer alone [5, 12]. So far, there were many kinds of keratinase produced from degraded feather microorganism, but there were very rare researches about it from wool-degrading microorganism [12, 13], and there were much fewer researches using it as the treatment process in improvement of shrink resistance of wool fabric [4]. We know that specificity is a main feature of enzyme. Keratinases, produced from microorganism using different keratin agar, could degrade different keratin [13]. Keratinase purified from *Pseudomonas* had been reported [14, 15], but this bacterial strain had been isolated using feather powder agar. In this research, we isolated a keratinase-producing bacterium strain, and it was identified as *Pseudomonas*. Because of the difference of isolated source with *P. aeruginosa* [14], the bacterium strain in this article possibly is not the same with other *Pseudomonas*. The difference was also shown by16S rRNA sequences.

Instead of protease process, keratinase process may improve shrink resistance of wool fabrics with only one step. The felting shrinkage of wool fabrics may reach 4.1% at 6 h through treatment of keratinase process of *Pseudomonas* sp. 3096-4 and lower than 5%, which accords with the standard of machine-washable IWTO TM31.

Another advantage of the process of keratinase treatment is that tensile strength of the treated wool fibers will be lost less and even increased. More weight and tensile strength are lost in the general protease process, and the main reason was that general protease process cannot degrade cuticle layers, and its permeation into the inner wool fiber damages the fiber. In this study, although polyester possibly mainly contributed to fabric tensile strength, tensile strength enhancing would be another reason. Keratinase could degrade cuticle layers, and it degraded wool fibers from outside to inside. Thus, wool fabric strength

did not decrease. Moreover, the peptide and amino acid that degraded from cuticle layers by keratinase diffused into the wool fibers and increased the tensile strength. In other studies [16, 17], the mixture of peptides and amino acid from keratin were used to treat damaged hair, and the mixture had a good affinity to hair and formed film on the surface of hair, thus peptides and amino acid from keratin not only repaired cuticle layer but also improved the luster and elasticity of the fiber. Yutaka et al. [18] reported that hydrolyzed keratin from waste wool or down were effective for enhancing the tensile properties of bast fibers. In our study, the treatment solution includes not only keratinase but also hydrolyzed keratin. Therefore, tensile strength of the treatment samples was not decreased.

In spite of these advantages, some potential issues need to be considered for the practical application. First, the result of keratinase's decreased wool fabrics' felting shrinkage by one step was obtained only in the laboratory, and the process of decreased wool fabrics' felting shrinkage in the industry should be further tested. Second, the processing time was long (6–15 h). To achieve a shortened treatment, some conditions including enzyme dose, addition metal ions, and other factors need to be optimized accordingly.

Conclusions

It is a tendency to use enzymes in the textile finishing industry. In this research, a new isolated bacterium showed excellent capability of removing cuticle layer of wool fibers, as demonstrated by removing cuticle layer completely within 48 h. It was classified as *Pseudomonas*. For the treatment with crude keratinase from this strain at optimized treatment, the wool fabrics' felting shrink was 4.1% at 6 h, and textile strength was not lost. As we have known, this is the first process of enzyme development used to improve anti-felting alone. This is a potential process to be used in wool fabric industry.

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