Heterologous Expression of Alkaline Metalloproteinases in Bacillus Subtilis SCK6 for Eco-Friendly Enzymatic Unhairing of Goatskins

by

Shihao Zhang, Ruoshi Zhang, Xiaoguang Li, Zhe Xu and Yongqiang Tian*

Key Laboratory of Leather Chemistry and Engineering, Ministry of Education, College of Biomass Science and Engineering, Sichuan University, No. 24 South Section 1, Yihuan Road, Chengdu 610065, People's Republic of China.

Abstract

In this study, alkaline metalloprotease gene 1067 was cloned from *Planococcus halotolerans* SCU63^T and heterologously expressed in *Bacillus subtilis* SCK6. Using Luria Bertani (LB) broth medium as the initial medium, the optimal medium was obtained through a series of fermentation and culture optimization (g/L): yeast extract (10), soybean powder (15), urea (20), potassium chloride (6.7), calcium chloride (13.3), NaCl (10). On the basis of the optimal medium, the highest enzymatic activity of 1259.21 U/mL could be obtained by culturing at 30°C for 40 h. with pH 8, inoculation amount of 4% and filling amount of 50 mL. EDTA inhibits protease activity and PMSF promotes it, indicating that it was a metalloprotease rather than a serine protease. The optimum reaction temperature of the protease is 70°C, and the optimum pH is 9. The metal ions Zn^{2+}, Co^{2+} and surfactant β-ME, Tween 80 can improve the activity of the protease. The results of unhairing, SEM and staining show that this metalloprotease can completely dehair goatskin. Compared with the conventional chemical method, the goatskin after enzymatic unhairing has softer texture and smoother surface, and there is no obvious damage to the goatskin.

Introduction

Leather industry is very popular at present, but it can also be a very polluting industry to the environment. Unhairing, one of the important operations in leather production, consists in removing the hairs of the hides and skins.[1] Conventional chemical unhairing process generates a lot of high pollution wastewater,[2, 3] solid waste[4] and large amounts of biochemical oxygen demand (BOD), chemical oxygen demand (COD), total suspended solids (TSS).[5-8] In addition, chromium (III) reagents used in chrome tanning and sodium sulfide used in unhairing have the potential to be converted to toxic chromium (VI) and hydrogen sulfides (H_2S).[9,10]

Due to above disadvantages, conventional chemical methods have been criticized by many environmentalists,[11] and people involved in the tanning industry are trying to find an alternative to conventional chemical methods for unhairing. In the past decades, it has been considered that the most promising alternative to conventional chemical unhairing methods is enzymatic unhairing.[12] At present, more and more enzymes are being used in leather industry, which include alkaline protease, neutral protease,[13] cellulase,[14] elastase,[15] α-amylase,[16] β-glucanase and so on. Alkaline proteases have the advantages of high catalytic activity,[17] strong substrate specificity[18] and high pH tolerance. Most alkaline proteases do not have keratinase activity, so the hair is relatively intact after enzymatic unhairing compared to the hair after conventional chemical unhairing.[19] In addition, alkaline conditions will make the hair roots swell and easy to dehair.[20] Alkaline proteases play an important role in both cell metabolism and biological activities, accounting for more than 65% of global sales of industrial enzymes.[21]

Enzymatic unhairing is a complex process. Protease hydrolyzes the proteoglycans near the hair root,[22-24] which reduces the bonding ability of hair and hair follicles, and loosens the elastic fibers of the collagen nearby,[25] effectively causing the loose surface of the skin. In addition, keratin of hair is hydrolyzed by keratinase, its disulfide bond is broken, with some oligomer and monomer products are produced.[26] However, enzymatic hydrolysis is not always beneficial since excessive collagen hydrolysis activity can lead to proteases attacking the collagen in the granular layer, causing damage of grain structure and its destruction, or even rotting of the skin.[27-29] Enzymatic unhairing process is often accompanied by hydrolysis of collagen.[30] Therefore, it is necessary to precisely control the amount of protease to avoid rotting of the skin, which needs to be improved in future enzymatic unhairing process.

Proteases come from a wide range of sources. Never *et al*. extracted alkaline protease from *Aspergillus oryzae* MG429773 and studied the effects of alkaline protease on unhairing under different conditions.[31] Madiha Shaikh *et al.* investigated the use of soil-derived *Streptomyces* in unhairing.[32] Zhou *et al*. cloned a novel serine basic protease (APRA) gene from Alkaliphilic *Idiomarina* sp. C9-1, which was expressed in *Escherichia coli* and used it for unhairing.[33] In addition, the proteases used for unhairing also come from other species such as *Pseudomonas aeruginosa*[15] *Conidiobolus* sp.,[34] *Doratomyces* sp.,[35] *Acinetobacter* sp.,[30] *Trichoderma* sp.[36] and *Caldicoprobacter* sp..[10]

In this study, a new gene fragment of metalloprotease from *Planococcus halotolerans* SCU63^T[37,38] and heterologously expressed in

*Corresponding author email: yqtian@scu.edu.cn

Manuscript received August 3, 2021, accepted for publication October 10, 2021.

Bacillus subtilis SCK6 was cloned, guaranteeing that no collagenases were expressed.[39] In addition, a series of unhairing experiments were carried out using the cloned protease, and it is expected that this protease can be widely used in leather industry.

Materials and Methods

Materials

The wet-salted goatskin was purchased from a local slaughterhouse in Chengdu. Both the heterologous expression host *Bacillus subtilis* SCK6 and the vector plasmid pWB980 were stored in College of Biomass Science and Engineering in Sichuan University. The 1067 metalloprotease gene fragment was obtained from *Planococcus halotolerans* SCU63^T stored in the same place as above. The high-fidelity enzyme (Phanta® Max Super-Fidelity DNA Polymerase) that constructed recombinant plasmid pWB980-1067 was purchased from Vazyme (Nanjing, China). Other analytical grade reagents were purchased from local suppliers in Chengdu.

Construction of recombinant plasmid and expression of 1067 in *B. subtilis* SCK6

As pWB980 is not a shuttle plasmid, the recombinant plasmid cannot be constructed by means of enzyme cleavage-ligation. Therefore, PCR technology (polymerase chain reaction) was needed to construct plasmid polymers through two PCR sequences to realize the connection between the protease gene 1067 and the expression plasmid pWB980.[40]

The gene of protease (1067) was amplified by PCR by primer 1067-pWB980F/1067-pWB980R, and the plasmid pWB980 was linearized by high-fidelity PCR by primer pWB980-1067F/pWB980-1067R. The above two products were purified by kit method (Extraction Mini Kit, Vazyme, Nanjing). The purified product was polymerized using a high-fidelity enzyme by PCR and identified by Nucleic Acid Electrophoresis. After that, the constructed recombinant plasmid was imported into *B. subtilis* SCK6 competent cells. The constructed recombinant bacteria were coated on LB solid medium containing 2% (w/v) nonfat powdered milk with 50 mg/L kanamycin at 37°C for overnight growth. A strain of positive clone was selected and grown in LB broth medium containing 50 mg/L kanamycin as seed cells. Then the seed cells were inoculated (2%, v/v) into LB broth medium with 50 mg/L kanamycin at 37°C, 220 rpm for 48 h.

Optimization of fermentation conditions

On the basis of LB broth medium, different kinds and concentrations of carbon source, nitrogen source, inorganic salt ions, as well as different pH, temperature, fermentation time, inoculation amount and liquid loading amount were selected to optimize the medium and culture conditions. The three factors with the highest influence on enzyme activity were selected for Response Surface Methodology (RSM). A three-factor and three-level analysis with enzyme activity as the response value was designed according to the Box-Behnken experimental principle by Design-Expert 12 software.

Purification of protease 1067

The fermentation broth was centrifuged at 7000 rpm, 4°C, for 10 min, and the supernatant was added with 60% (w/v) ammonium sulfate and placed at 10°C overnight to precipitate the protein. The protein precipitates were redissolved in buffer (50 mM Tris-HCl, pH 7.0) and dialyzed in an 8000D dialysis bag for 24 h. During this period, the dialysis solution was changed every 4 h. The enzyme solution in the dialysis bag was centrifuged at 10000 rpm, 10°C for 10 min to obtain the supernatant. The supernatant was chromatographed on a Q Sepharose Fast Flow (GE healthcare, USA) column which was balanced with buffer A (50 mM Tris-HCl, pH 7.0), and eluted with a NaCl (0-1M) linear gradient. Proteins eluted at different NaCl concentrations were identified by SDS-PAGE of 12.5%.

Enzyme assay

The activity of partially purified crude enzymes was determined by Folin-Phenol method.[41] The mixture (250 mL) of enzyme and buffer solution were mixed with 250 mL casein (2%, w/v, pH 7.5), and after 20 min of reaction at 40°C, 500 mL trichloroacetic acid (TCA, 0.4 M) was added to terminate the reaction. The mixed liquid was centrifuged at 12000 rpm for 10 min. Then 500 μL supernatant, 2.5 mL sodium carbonate (0.4 M) and 500 μL folin-phenol (1 M, Solarbio) were mixed evenly and reacted at 40°C for 20 min. The absorbance was determined at OD_{680} with the control group as blank. The order of adding casein and TCA in the control group was opposite to that in the experimental group. However, the other steps were the same as those in the experimental group.

Analysis of enzymatic properties

The properties of protease 1067 are affected by various environmental factors such as temperature, pH, NaCl concentration, surfactants, organic solvents, and chemical reagents.

In this experiment, different temperatures (30°C to 80°C in 5°C increments) were used to test the activity and stability of the protease. The protease was reacted at the itemized temperatures, and the activity of the protease was detected after 30 min incubation at the corresponding temperature to study the thermal stability of the protease.

The pH value reflects the acid-base environment in which the protease is located, and different pH values have different effects on the protease. In this experiment, different pH values (pH 2-4, Glycine-HCl; pH 5-6, NaAc-HAc; pH 7-9, Tris-HCl; pH 10-11, Glycine-NaOH; pH 12-13, KCl-NaOH) were selected to study the influence of pH on protease activity and stability.

In order to study the effect of metal ions on protease activity, different metal ions (Mn^{2+}, Zn^{2+}, Co^{2+}, Sr^{2+}, Ni^{2+}, Ba^{2+}, Fe^{3+}, Mg^{2+}, Ca^{2+}, K^{+},

Cd^{2+}, Cu^{2+}, Cr^{3+}, Fe^{2+}, Ag^{+}, Li^{+}, Cr^{6+}) were selected and reacted with the protease solution using similar method as described above.

Similarly, different chemicals (PMSF - Phenylmethylsulfonyl fluoride, DMSO - Dimethyl sulfoxide, β-ME - 2-Hydroxy-1-ethanethiol, SDS - Sodium dodecyl sulphate, EDTA - Ethylenediamine tetraaceticacid, Tween 20, Tween 80, DTT – Dithiothreitol, TritonX-100, EGTA, GmHCl - Guanidine Hydrochloride , Urea, H_2O_2) and organic solvents (formamide, glycerin, n-hexane, acetone, isopropanol, ethylene glycol, methanol, benzene, ethanol, n-butanol, isoamyl alcohol) were used to study their effects on protease activity.

Unhairing function of protease 1067

The wet-salted goatskin was cut into 4.5 cm*4.5 cm square shapes with the weight of each skin at about 3.5 ± 0.5 grams. The pieces were washed with clean water to remove blood stains, sand, preservatives and other insoluble substances. Nine goatskins were divided into three groups and each group was subjected to dehairing experiments using different methods, named 1067, 1398 and the control group. The washed goatskin pieces were placed in a triangular flask; one containing 50 mL of 1067 crude protease, while the second contained sodium sulfide solution (3%, w/v) and 50 mM Tris-HCl (pH 7.0) was used in the control group instead of the protease solution at 37°C, 220 rpm for unhairing in conventional chemical method. The 1398 group used 50u of protease for unhairing experiment and other conditions were the same as 1067 group.

Characterization of protease 1067 unhairing

The content of substances in the wastewater after unhairing is an important index to detect the environmental protection of unhairing. The environmental friendliness of different unhairing methods was studied by detecting the contents of chloride, ammonia nitrogen, BOD, COD and TSS in wastewater.

After unhairing, the goatskins were cleaned and divided into small pieces. The washed goatskin was immobilized in tissue fixation solution (4% paraformaldehyde, PB buffer, Wuhan) for 36 h. The fixed tissue was embedded in paraffin and sliced with a frozen slicer. Elastic fibers were stained by H&E-staining and collagen fibers were stained by Masson-staining.[42] And the microscope was used to observe the staining results.

The cleaned and dehaired goatskin sections were refrigerated and then put into a freeze-dryer to remove the moisture through sublimation. Then scanning electron microscopy (SEM) was used to take photos of the treated goatskin sections to check the unhairing effect of different unhairing methods.

Wet blue skin was obtained by dipping, deliming, softening, acid dipping, chrome tanning and other processes after unhairing in the drum.[43] The tensile strength, tear strength and bursting strength of wet blue leather were measured to characterize its mechanical properties.

Results and Discussion

Construction of recombinant strain and identification of protease activity

The successfully constructed recombinant strain was identified using PCR by primers 1067F/1067R which were designed according to the position of the inserted fragment in the recombinant plasmid. According to the genomic information of SCU63, the full length of metalloprotease 1067 gene was 1662 bp, and the full length of pWB980 vector was 3787 bp. The construction results of plasmid polymer and the verification results of recombinant bacteria are shown in Figure 1a. The size of the gene fragment was between 1000~2000 bp, which was consistent with the genomic information. The molecular weight of plasmid polymers is much larger than 5000 bp, which is in line with the size range of plasmid polymers. The recombinant bacteria were evenly coated on solid LB broth medium containing 2% (w/v) nonfat milk powder and 50 mg/L kanamycin for overnight growth, and the enzyme activity was determined by the presence or absence of proteolytic circles. As shown in Figure 1b, it can be seen that there are obvious proteolytic circles around the bacteria.

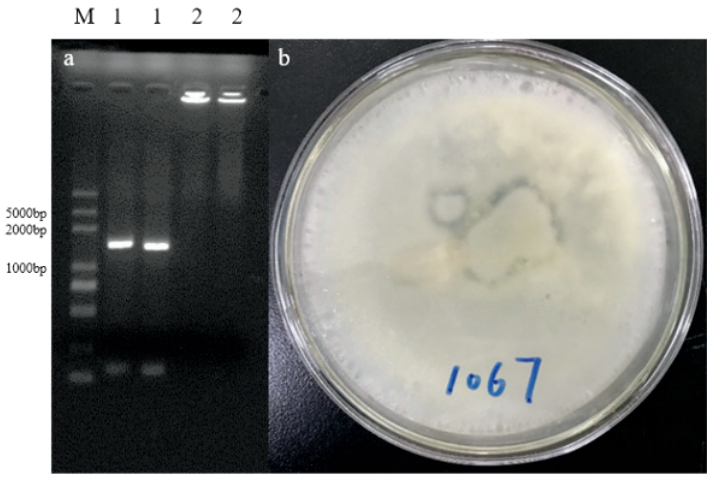

Figure 1. Construction of plasmid polymers and expression of protease. **a:** Construction of recombinant plasmid and verification of successful introduction of protease gene, M: marker, 1: metalloprotease 1067 gene fragment, 2: plasmid polymers; **b:** Hydrolytic circle of protease 1067

Fermentation optimization

According to the Luria Bertani (LB) broth medium (Casein Tryptone of 1%, Yeast extract 0.5%, NaCl of 1%, pH 7.0), the optimal fermentation conditions were obtained according to a series of fermentation optimizations (w/v): yeast extract (1%), soybean powder (1.5%), urea (2%), potassium chloride (0.67%), calcium chloride (1.33%), inoculation amount (4%, v/v), liquid filling amount (50 mL), pH (8.0), temperature (30°C), fermentation time (40 h). Through the optimization of fermentation conditions, the enzyme activity reached the highest 1259.11 U/mL from the initial 8.74 U/mL.

As shown in Table I, three conditions of carbon source, nitrogen source and pH were selected for Response Surface Methodology (RSM). As can be seen from Table II, both p-value of 0.0135 < 0.05 and F-value of 6.04 indicate that the quadratic equation model is significant. Lack of fit is 0.1305 > 0.05, indicating that model is not significant and the sub-model fits well in the regression region. It can be interpreted from the table that among the three factors, carbon source is the factor that has the greatest influence on enzyme activity, followed by pH value and nitrogen source.

It can be seen from the three response surface curves in Figure 2 that each response surface is a convex surface, indicating that this model has a stability point within the experimental range. From the perspective of the projection (the contour line) of the 3D surface, the pair interaction of the three factors presents an ellipse, which represents the significant interaction effect between the two factors.

Table I

Factors and Levels for RSM

Level	Factor		
	A-carbon source	B-nitrogen source	C-pH
-1	5	10	7.5
0	10	15	8
1	15	20	8.5

Table II

ANOVA for Quadratic Model

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	153.12	9	17.01	6.04	0.0135	significant
A-Carbon source	17.13	1	17.13	6.08	0.043	
B-Nitrogen source	1.6	1	1.6	0.5692	0.4752	
C-pH	3.9	1	3.9	1.39	0.2776	
AB	6.26	1	6.26	2.22	0.1796	
AC	2.25	1	2.25	0.7973	0.4015	
BC	10.08	1	10.08	3.58	0.1004	
A^2	84.63	1	84.63	30.05	0.0009	
B^2	7.35	1	7.35	2.61	0.1502	
C^2	11.93	1	11.93	4.24	0.0786	
Lack of Fit	14.23	3	4.74	3.47	0.1305	not significant

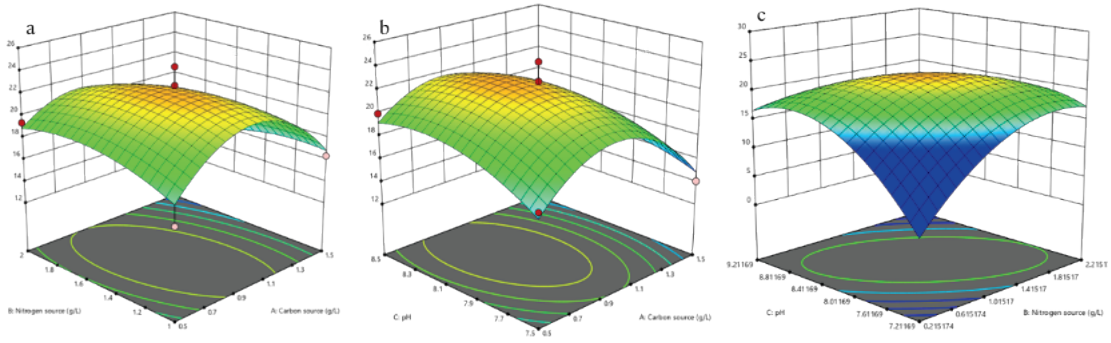

Figure 2. 3D surface of three-level and three-factor response surface.

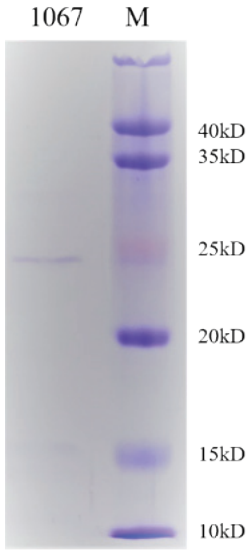

Figure 3. SDS-PAGE of purified protease 1067. M: Marker, 1067: protease 1067

Purification and identification of protease 1067

The crude protease was purified by centrifugation, precipitation, and Q Sepharose Fast Flow. The molecular weight was determined to be 25.04 kD by 12.5% SDS-PAGE (Figure 3).

Effect of temperature on enzyme activity and stability

As shown in Figure 4a, enzyme activity increased very slowly between 30°C and 40°C, then increased rapidly from 40°C to 70°Cand reached the maximum at 70°C. After 70°C, enzyme activity decreases with increasing temperature. When the temperature reached 85°C, the enzyme activity was only 20% of that at 70°C. Figure 4b shows the effect of temperature on the stability of the enzyme. The enzyme activity remained above 75% of the maximum enzyme activity between 30°C and 50°C, and the remaining enzyme activity was the highest when incubated at 40°C for 30 min. As the temperature continues to increase, the enzyme activity continues to decrease, and the enzyme activity can hardly be detected at 85°C. The results showed that the protease 1067 had good thermal stability in the range of 30°C to 50°C, and 70°C was the most suitable reaction temperature. Its optimum reaction temperature is higher than that of keratinase gene in *Bacillus subtilis*[1] and kerT in WB600.[44]

Effect of pH on enzyme activity and stability

As shown in Figure 5a, the activity of enzyme increased from pH 2 to 9 and reached its maximum at pH 9, and after pH 9, the enzyme activity began to decline rapidly. When the pH was 13, the relative enzyme activity was almost zero. The protease 1067 maintains high activity at pH 4 to 9 and low activity at more acidic or alkaline

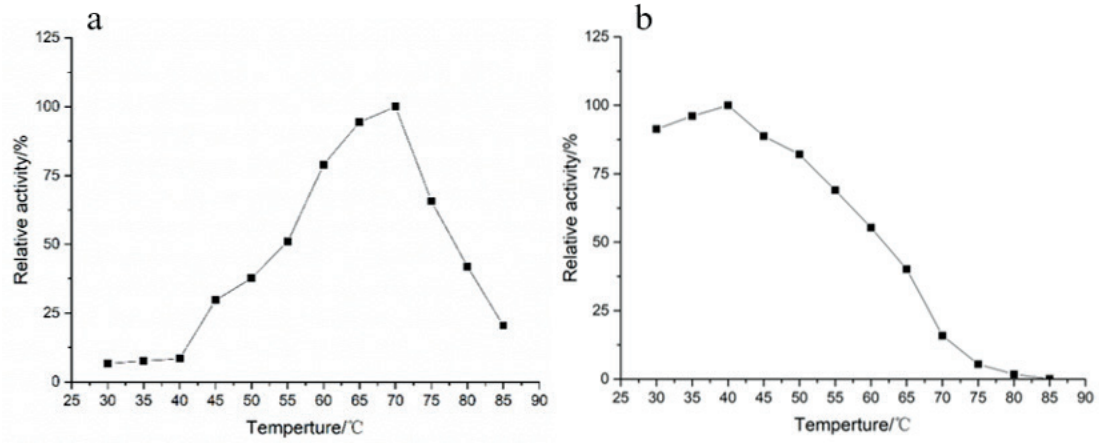

Figure 4. Effect of temperature on enzyme activity and stability. **a:** effect of temperature on enzyme activity; **b:** effect of temperature on enzyme stability

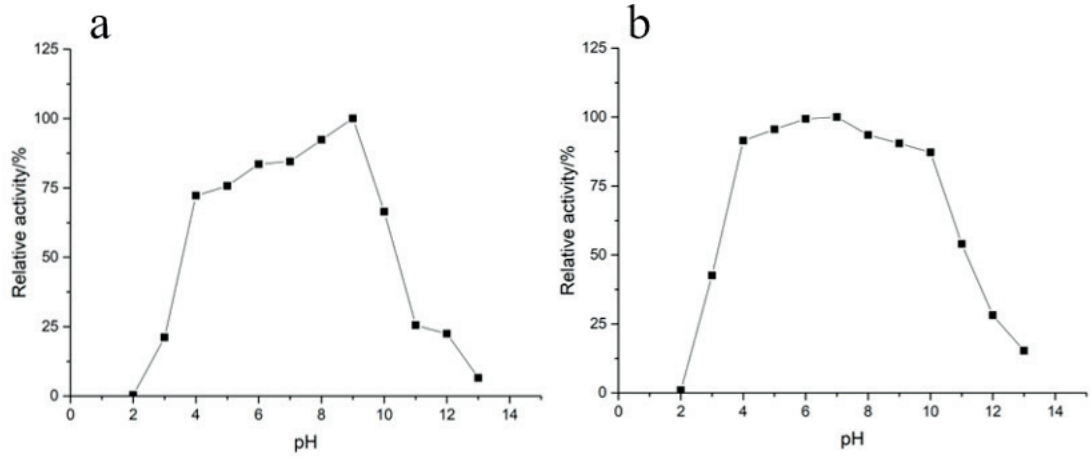

Figure 5. Effect of pH on enzyme activity and stability. **a:** effect of pH on enzyme activity; **b:** effect of pH on enzyme stability

conditions. As shown in Figure 5b, the protease 1067 has a broad pH tolerance between pH 4 and 10. Besides that, it is highly active at pH 4 to 10 and the highest enzyme activity was obtained in a buffer of pH 9.0, indicating that it is an alkaline protease and stable over a wide range of pH values. The conditions of action of this alkaline protease are milder than those of other alkaline proteases and have less impact on the environment.[1]

Table III

Effect of metal ions on enzyme activity

Metal ions	Relative activity (%)
Control	100.00 ± 0.19
Mn^{2+}	96.73 ± 0.09
Zn^{2+}	118.64 ± 0.06
Co^{2+}	124.27 ± 0.27
Sr^{2+}	94.67 ± 0.07
Ni^{2+}	86.46 ± 0.15
Ba^{2+}	91.55 ± 0.25
Fe^{3+}	72.07 ± 0.02
Mg^{2+}	92.70 ± 0.08
Ca^{2+}	99.72 ± 0.27
K^{+}	36.23 ± 0.01
Cd^{2+}	42.47 ± 0.02
Cu^{2+}	79.38 ± 0.01
Cr^{3+}	61.27 ± 0.15
Fe^{2+}	80.29 ± 0.06
Ag^{+}	94.45 ± 0.08
Li^{+}	91.93 ± 0.09
Cr^{6+}	80.14 ± 0.10

Effect of metal ions on enzyme activity

The influence of metal ions (concentration of 5 mM) on enzyme activity can be clearly seen from Table III. Most metal ions such as Mn^{2+}, Sr^{2+}, Mg^{2+}, and Ag^{+} have a slight inhibitory effect on enzyme activity while Ni^{2+}, Cu^{2+}, Fe^{2+}, and Cr^{6+} have a great inhibitory influence on enzyme activity. The enzyme activity was significantly inhibited by K^{+} and Cd^{2+}, and the enzyme activity was only 36.23 ± 0.01% and 42.47 ± 0.02% of the control group. Only Zn^{2+} and Co^{2+} significantly promoted the enzyme activity, which reached 118.64 ± 0.06% and 124.27 ± 0.27% of the control group, respectively. There was a significant increase in enzyme activity in the presence of Zn^{2+}, which verified that the protease belongs to the zinc-dependent metalloprotease family.[45] The experiments of Tian *et al.* also found that Co^{2+} had some promotion effect on protease.[43] In addition, the concentration of Co^{2+} also affects enzyme activity, Zhang *et al.* showed that low concentrations of Co^{2+} promoted enzyme activity, while high concentrations of Co^{2+} had a significant inhibitory effect on enzyme activity.[30]

Effect of chemicals on enzyme activity

As shown in Table IV, EDTA completely inhibited the activity of the enzyme, while PMSF did not inhibited but promoted the activity of the enzyme, indicating that the protease 1067 was a metalloprotease rather than a serine protease.[46] Surfactants SDS at concentrations of 10% inhibited the enzyme activity, which was only 76.66 ± 0.06% of that of the control group. Surfactants Tween 20 (10%) and TritonX-100 (1%) had no significant effect on enzyme activity, while Tween 80 (1%) and β-ME (10 mM) increased enzyme activity to 111.21 ± 0.06% and 162.05 ± 1.76% of the control group.

Table IV

Effect of chemicals on enzyme activity

Chemicals	Concentration	Relative activity (%)
Control		100.00 ± 0.06
PMSF	10 mM	96.20 ± 0.03
DMSO	10%	79.70 ± 0.36
β-ME	10 mM	162.05 ± 1.76
SDS	10%	76.66 ± 0.06
EDTA	10 mM	0.00 ± 0.00
Tween 20	10%	98.68 ± 0.14
Tween 80	1%	111.21 ± 0.06
DTT	10 mM	89.55 ± 0.11
TritonX-100	1%	102.61 ± 0.16
EGTA	10 mM	61.98 ± 0.01
Urea	0.5 M	100.57 ± 0.18
H_2O_2	1%	95.16 ± 0.08
GmHCl	0.5 M	90.03 ± 0.09

Table V
Effect of solvents on enzyme activity

Solvents	Relative activity (%)
Control	100.00±0.06
Formamide	95.52±0.04
Glycerol	90.94±0.08
Hexyl hydride	81.79±0.06
Acetone	76.26±0.06
Isopropanol	98.67±0.05
Ethylene glycol	86.52±0.03
Methanol	87.15±0.04
Benzene	85.26±0.06
Ethanol	92.99±0.02
N-Butanol	83.68±0.01
Isopentanol	98.36±0.11

Higher concentrations of β-ME than in this experiment were also shown to have a promotive effect on enzyme activity.[45] EGTA had a strong inhibitory effect on the enzyme activity, at 61.98 ± 0.01% of the control group. Similar results were obtained by Zhou *et al.* who used different concentrations (2mM, 5mM, 10mM) of EGTA and all results showed an inhibition of enzyme activity.[33]

Effect of organic solvents on enzyme activity

The influence of the organic solvents (10%) selected for this experimental study on enzyme activity is shown in Table V. All of the selected solvents have more or less inhibitory effect on enzyme activity. Acetone has the strongest inhibitory effect on enzyme activity, which was 76.26 ± 0.06% of the control group. Isopentanol, isopropanol and formamide had little to no effect on enzyme activity. The above experimental results suggest that it may have excellent application in non-aqueous phase protease catalysis.[47] The tolerance of proteases to organic solvents indicates their potential for application in non-aqueous phase mixed solutions.[45] It has been shown that the higher the concentration of organic solvents, the greater the inhibitory effect on enzyme activity.[48] The protease in this experiment was well tolerated in some organic solvents and has some potential for non-aqueous phase catalytic applications.

Analysis of unhairing

As shown in Figure 6, enzymatic unhairing can be achieved completely. Sensory tests showed that the goatskin after enzymatic unhairing was smoother, whiter and softer than those after conventional chemical unhairing. In addition, after enzymatic unhairing, the epidermis of goatskin was completely removed, and there was no obvious damage to the skin in 6 h. The results of stereomicroscopy showed that after unhairing under the action of protease 1067, the goatskin had clear pores and no residual hair. Nevertheless, after unhairing with sodium sulfide, the goatskin had no obvious pores, while after protease AS1.398 depilation, skin had residual hair. The results of unhairing experiment showed that enzymatic unhairing had the same ability of complete unhairing as conventional chemical method, and the unhairing effect was better, which was also confirmed by sensory analysis.

The content of main substances in wastewater after depilation is shown in Figure 7. It can be seen that the BOD, COD, TSS, and ammonia nitrogen in the wastewater after the unhairing of protease

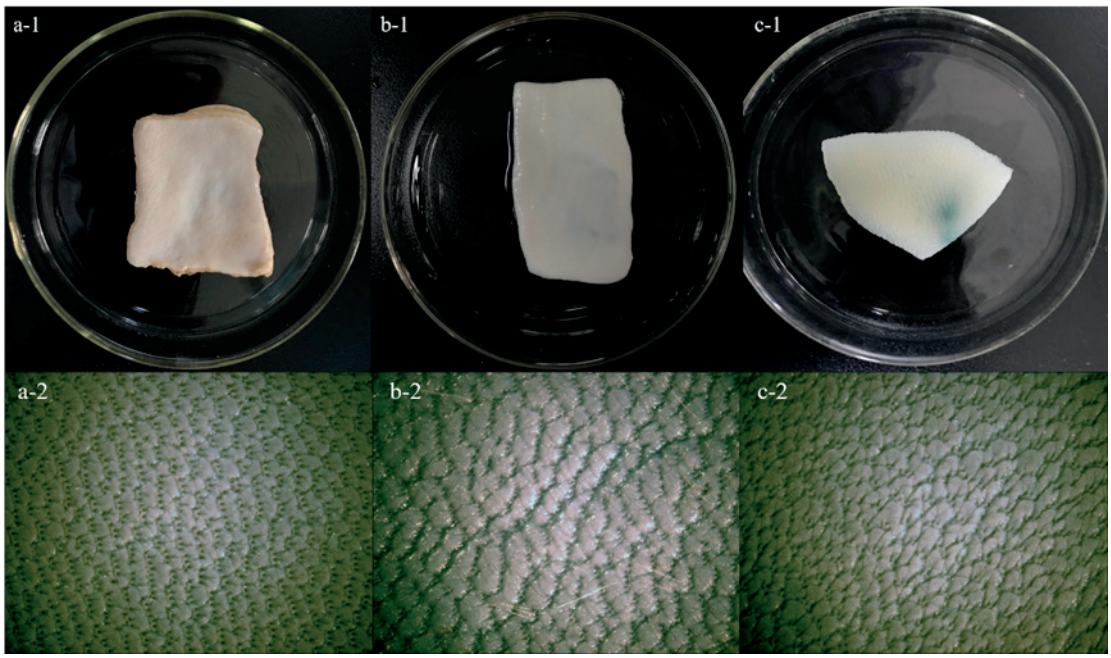

Figure 6. 1: Unhairing performance on goatskins by different unhairing methods.
2: The grain surface of pelts with different unhairing methods.
a: unhairing by protease 1067; **b:** unhairing by protease AS1.398; **c:** unhairing by Na_2S

1067 are significantly less than those of the conventional chemical method and protease AS1.398 unhairing. Compared with protease AS1.398 unhairing method, the contents of BOD, COD, TSS and ammonia nitrogen after unhairing of protease 1067 decreased to 22.27%, 21.39% and 17.05%, which were 16.96%, 15.59%, 10.78% and 62.56% of conventional chemical unhairing respectively.

SEM and analysis of tissue staining

The scanning electron microscopy (SEM) was employed to observe the cross-section conformation of dehaired goatskin samples (Figure 8). The reticular fibers of goatskin after enzymatic unhairing (Figure 8a) were more dispersed and uniform, while the reticular fibers of goatskin after conventional chemical unhairing (Figure 8b) were not uniform. This result was also consistent with the conclusion drawn by Parthiban *et al.* reporting that the fiber loosening effect of enzymatic unhairing was better than the chemical unhairing.[18]

H&E-staining and Masson-staining methods were used to stain and observe the section of naked skin after unhairing, and the results are depicted in Figure 9. The staining images confirmed the presence of hair roots, hair follicles, glands, and other structures in the goatskin treated with Na_2S, while no hair roots, glands, and other structures were observed in the goatskin treated with protease 1067. There are still hairs left in the hair follicle after unhairing by protease AS1.398,

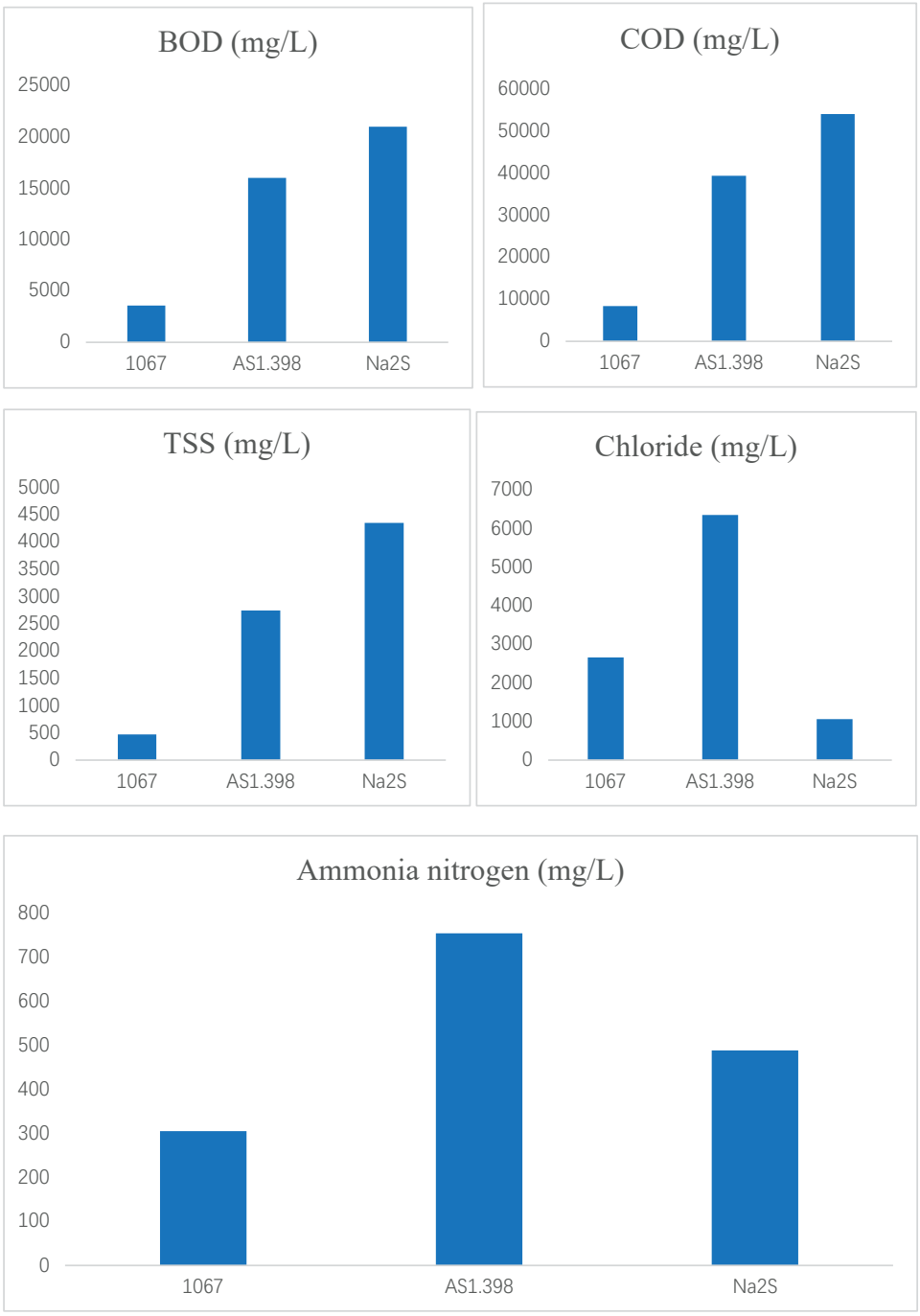

Figure 7. The content of main substances in the wastewater after unhairing

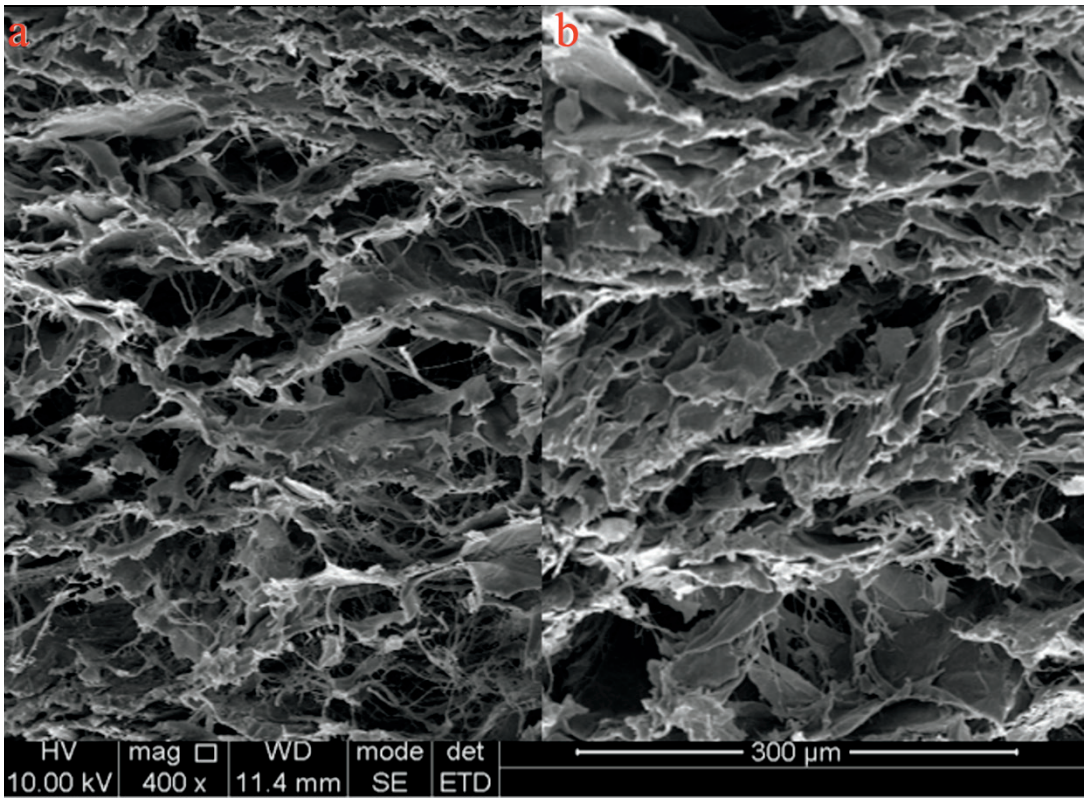

Figure 8. The cross section of dehaired skin detected by SEM.
a: enzymatic unhairing; **b:** conventional chemical unhairing

which is not as effective as protease 1067. Compared with chemical unhairing method, the staining results of skin tissue after enzymatic unhairing showed that the red was lighter and the blue was darker, which indicated that the non-collagen composition was reduced, and the leather quality could be improved. Goatskin treated with protease 1067 has similar softness and better unhairing effect as protease AS1.398. In general, protease 1067 can remove hair roots, glands, and other structures and disperse collagen fibers. Moreover, there was no obvious epidermis on the surface of the skin after protease treatment, indicating that the protease could hydrolyze the epidermis.

Table VI summarizes the mechanical properties of wet blue skin after unhairing by different methods. In the tensile strength index, although the strength of wet blue skin after unhairing with protease 1067 was lower, the elongation rate was the best, which was significantly better than that of chemical and protease AS1.398 unhairing methods. The tear strength characterization showed that the strength of wet blue skin after protease 1067 unhairing was higher than that of protease AS1.398. Among the three methods, the best burst strength of wet blue skin after unhairing with protease 1067 was 406.103 N/mm. Studies have also confirmed that protease unhairing is effective in improving the performance of leather.[42, 49]

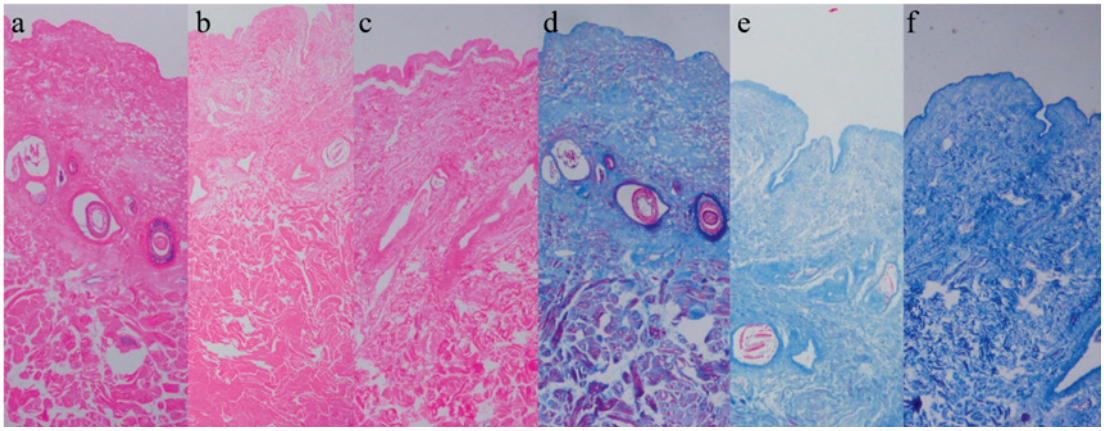

Figure 9. Staining results of bare skin. **a, b, c:** The results of H&E-staining of goat skin treated with Na_2S, AS1.398 and 1067; **d, e, f:** The results of Masson-staining of goat skin treated with Na_2S, AS1.398 and 1067

Table VI

Characterization of mechanical properties of wet blue skin

	Tensile strength		Tearing strength	Burst strength
	Tensile strength (N/mm^2)	Elongation at break (%)	Tearing strength (N/mm)	Burst strength (N/mm)
1067	17.805	68.000	64.213	406.103
AS1.398	24.992	46.500	41.506	312.246
Na_2S	27.888	40.165	82.177	281.730

Conclusion

Conventional chemical unhairing is a very polluting process. In order to reduce the environmental pollution caused by unhairing and to maintain the sustainability of the leather industry, this study developed an alkaline protease that can act on the unhairing of goatskins.

In this study, an alkaline metalloprotease gene (1067) was cloned from *Planococcus halotolerans* SCU63^T and transferred into *Bacillus subtilis* SCK6 for heterologous expression. After a series of optimization experiments, and the enzyme activity increased from the initial 8.74 U/mL to 1259.11 U/mL. The enzymatic properties of the purified enzyme after fermentation were found that Zn^{2+}, Co^{2+} Tween 80, β-ME significantly enhances the activity of the enzyme, and the protease is also tolerant to low concentrations of organic solvents. In addition, enzymatic unhairing experiments have proven that, compared to conventional chemical hair removal methods, the enzymatic unhairing method has good results on goatskin. The unhairing effect was further analyzed by SEM, H&E-staining and Masson-staining, and the results of above methods showed that this metalloprotease had the ability to completely remove hair of goatskin, the softness and whiteness of goatskin after enzymatic unhairing are excellent, and the quality of the leather is also excellent. After testing, the contents of BOD, COD, TSS, Chloride and Ammonia nitrogen in the wastewater produced by unhairing are significantly reduced, which has a very good effect on the environment.

In conclusion, this study has identified a protease for the unhairing process, which can effectively reduce the environmental pollution caused by unhairing, and it has promising applications in the future of unhairing process.

Acknowledgements

This work was supported by the National Key Research and Development Program of China (2017YFB0308401), and Sichuan Science and Technology project (2020JDPT0018).

References

1. Cao S., et al.; A novel unhairing enzyme produced by heterologous expression of keratinase gene (kerT) in Bacillus subtilis. *World J Microbiol Biotechnol*, **35**, 122, 2019.
2. Saravanabhavan Subramani, et al.; Reversing the Conventional Leather Processing Sequence for Cleaner Leather Production. *Environmental Science & Technology*, **40**, p.1069-1075, 2006.
3. Dayanandan A., et al.; Solid State Bioprocessing for Scale up of Aspergillus tamarii MTCC5152 Lipase and its Degreasing Effect on Cow Hide. *Indian Journal of Science and Technology,* **5**, 2978-2983, 2012.
4. Kanagaraj J., et al.; Eco-friendly waste management strategies for greener environment towards sustainable development in leather industry: a comprehensive review. *Journal of Cleaner Production*, **89**, 1-17, 2015.
5. Zarai Jaouadi N., et al.; Probing the crucial role of Leu31 and Thr33 of the Bacillus pumilus CBS alkaline protease in substrate recognition and enzymatic depilation of animal hide. *PLoS One*, **9**, e108367, 2014.
6. Ramesh R. R., Muralidharan, V., and Palanivel, S.; Preparation and application of unhairing enzyme using solid wastes from the leather industry-an attempt toward internalization of solid wastes within the leather industry. *Environ Sci Pollut Res Int*, **25**, 2121-2136, 2018.
7. Dettmer A., et al.; Optimization of the unhairing leather processing with enzymes and the evaluation of inter-fibrillary proteins removal: an environment-friendly alternative. *Bioprocess Biosyst Eng*, **35**, 1317-24, 2012.
8. Saranya R., et al.; Value addition of fish waste in the leather industry for dehairing. *Journal of Cleaner Production*, **118**, 179-186, 2016.
9. Dettmer A., et al.; Optimization of the Biotechnological Process for Hide Unhairing in Substitution of Toxic Sulfides. *Chemical Engineering & Technology*, **35**, 803-810, 2012.
10. Bouacem K., et al.; Novel serine keratinase from Caldicoprobacter algeriensis exhibiting outstanding hide dehairing abilities. *Int J Biol Macromol*, **86**, 321-8, 2016.
11. Shakilanishi S. and Shanthi, C.; Specificity studies on proteases for dehairing in leather processing using decorin as model conjugated protein. *Int J Biol Macromol*, **103**, 1069-1076, 2017.
12. Dettmer Aline, et al.; Environmentally friendly hide unhairing: enzymatic hide processing for the replacement of sodium sulfide and delimig. *Journal of Cleaner Production*, **47**, 11-18, 2013.
13. Lopez L. M. I., et al.; Latex peptidases of Calotropis procera for dehairing of leather as an alternative to environmentally toxic sodium sulfide treatment. *Bioprocess Biosyst Eng*, **40**, 1391-1398, 2017.
14. Y. Zhang, Z. Tang, H. Liu1, K. Tang; Effects of different salt-enzymes on opening up of collagen fiber bundles for leather making - 230.pdf, *IULTCS Poster Session #4*, 2019.

15. Jose D., Mohandas, A., and Singh, I. S. Bright %J Indian Journal of Biotechnology; Bioprocess technology for LasB protease (elastase) production from Pseudomonas aeruginosa MCCB 123. *Indian Journal of Biotechnology*, **17**, 284-289, 2018.
16. Pandi Ajitha, et al.; Inexpensive α-amylase production and application for fiber splitting in leather processing. *RSC Advances*, **6**, 33170-33176, 2016.
17. Wang C., et al.; Extracellular Proteome Profiling of Bacillus pumilus SCU11 Producing Alkaline Protease for Dehairing. *J Microbiol Biotechnol*, **26**, 1993-2005, 2016.
18. Sujitha P., et al.; Enzymatic dehairing: A comprehensive review on the mechanistic aspects with emphasis on enzyme specificity. *Int J Biol Macromol*, **118**, 168-179, 2018.
19. Gupta R. K., et al.; Scale-up of an alkaline protease from Bacillus pumilus MTCC 7514 utilizing fish meal as a sole source of nutrients. *J Microbiol Biotechnol*, **22**, 1230-6, 2012.
20. Gupta R., Beg, Q. K., and Lorenz, P.; Bacterial alkaline proteases: molecular approaches and industrial applications. *Appl Microbiol Biotechnol*, **59**, 15-32, 2002.
21. Rao, M B et al. Molecular and biotechnological aspects of microbial proteases. *Microbiology and molecular biology reviews*, **62**,3, 597-635, 597-635, 1998.
22. Cantera C. S., et al.; Hair Saving Unhairing Process. Part 5 Characterisation Of Enzymatic Preparations Applied In Soaking And Unhairing Processes. *JALCA* **87**, p.69-77, 2003.
23. Cantera C. S., et al.; Hair saving unhairing process: Part 6 stratum corneum as a diffusion barrier: Chemical-mechanical injury of epidermis. *JALCA* **88**, 121-131, 2004.
24. Jian S., Wenyi, T., and Wuyong, C.; Studies on the application of ultrasound in leather enzymatic unhairing. *Ultrason Sonochem*, **17**, 376-82, 2010.
25. Jian Song, Wenyi, Tao, and Wuyong, Chen; Kinetics of enzymatic unhairing by protease in leather industry. *Journal of Cleaner Production*, **19**, 325-331, 2011.
26. Jaouadi Nadia Zaraî, et al.; The Promising Keratin-Biodegradation and Hide-Dehairing Activities of the Keratinase KERUS from Brevibacillus Brevis Strain US575, Recent Advances in Environmental Science from the Euro-Mediterranean and Surrounding Regions. 2018. p. 133-135.
27. Choudhary R. B., Jana, A. K., and Jha, M. K.; Enzyme technology applications in leather processing. *Indian Journal of Chemical Technology,* **11**, 659-671, 2004.
28. Macedo Alexandre J, et al.; Novel Keratinase from Bacillus subtilis S14 Exhibiting Remarkable Dehairing Capabilities. *Applied and Environmental Microbiology* **71**, 594-596, 2005.
29. Li Y., et al.; A new approach for quantitative characterization of hydrolytic action of proteases to elastin in leather manufacturing. *Appl Microbiol Biotechnol*, **102**, 10485-10494, 2018.
30. Zhang R. X., et al.; A metallo-keratinase from a newly isolated Acinetobacter sp. R-1 with low collagenase activity and its biotechnological application potential in leather industry. *Bioprocess Biosyst Eng*, **39**, 193-204, 2016.
31. Never Zekeya, et al.; Dehairing of animal hides and skins by alkaline proteases of Aspergillus oryzae for efficient processing to leather products in Tanzania. *African Journal of Biotechnology*, **18**, 426-434, 2019.
32. Shaikh Madiha, et al.; Streptomyces sp. MM-3 from rhizosphere of Psidium guajava: a potential candidate for protease with dehairing properties. *Pakistan Journal of Botany*, **51**, 2019.
33. Zhou C., et al.; A novel alkaline protease from alkaliphilic Idiomarina sp. C9-1 with potential application for eco-friendly enzymatic dehairing in the leather industry. *Sci Rep*, **8**, 16467, 2018.
34. Khandelwal Harish B., et al.; Eco-friendly enzymatic dehairing of skins and hides by C. brefeldianus protease. *Clean Technologies and Environmental Policy*, **17**, 393-405, 2014.
35. Friedrich J., et al.; In vitro degradation of porcine skin epidermis by a fungal keratinase of Doratomyces microsporus. *Enzyme and Microbial Technology*, **36**, 455-460, 2005.
36. Ismail Abdel-Mohsen S., et al.; Novel keratinase from Trichoderma harzianum MH-20 exhibiting remarkable dehairing capabilities. *International Biodeterioration & Biodegradation*, **70**, 14-19, 2012.
37. Gan L., et al.; Genomic insights into the salt tolerance and cold adaptation of Planococcus halotolerans SCU63(T). *Arch Microbiol*, **202**, 2841-2847, 2020.
38. Gan L., et al.; Planococcus halotolerans sp. nov., isolated from a saline soil sample in China. *Int J Syst Evol Microbiol*, **68**, 3500-3505, 2018.
39. Tian Jiewei, et al.; Eco-friendly enzymatic dehairing of goatskins utilizing a metalloprotease high-effectively expressed by Bacillus subtilis SCK6. *Journal of Cleaner Production*, **212**, 647-654, 2019.
40. You C., Zhang, X. Z., and Zhang, Y. H.; Simple cloning via direct transformation of PCR product (DNA Multimer) to Escherichia coli and Bacillus subtilis. *Appl Environ Microbiol*, **78**, 1593-5, 2012.
41. Li Xia, et al.; Investigation of collagen hydrolysate used as carbon and nitrogen source in the fermentation of Bacillus pumilus. *Process Biochemistry*, **55**, 11-16, 2017.
42. Sivasubramanian S., et al.; Ecofriendly lime and sulfide free enzymatic dehairing of skins and hides using a bacterial alkaline protease. *Chemosphere*, **70**, 1015-24, 2008.
43. Tian J., et al.; High-expression keratinase by Bacillus subtilis SCK6 for enzymatic dehairing of goatskins. *Int J Biol Macromol*, **135**, 119-126, 2019.
44. S. Cao1, et al.;Development and Investigation of Low Collagen Degradability Unhairing Enzyme by Gene Modification.pdf. IULTCS Poster Session #1, 2019
45. Rehman R., et al.; Catalytic Role of Thermostable Metalloproteases from Bacillus subtilis KT004404 as Dehairing and Destaining Agent. *Appl Biochem Biotechnol*, **181**, 434-450, 2017.
46. Jagadeesan Yogeswaran, et al.; Sustainable production, biochemical and molecular characterization of thermo-and-solvent stable alkaline serine keratinase from novel Bacillus pumilus AR57 for promising poultry solid waste management. *International Journal of Biological Macromolecules*, **163**, 135-146, 2020.
47. Sareen R. and Mishra, P.; Purification and characterization of organic solvent stable protease from Bacillus licheniformis RSP-09-37. *Appl Microbiol Biotechnol*, **79**, 399-405, 2008.
48. M. Fathi Najafi, et al.; Potential application of protease isolated from Pseudomonas aeruginosa.pdf. *Electronic Journal of Biotechnology,* **8**(2) 2005.
49. Dayanandan A., et al.; Application of an alkaline protease in leather processing: an ecofriendly approach. *Journal of Cleaner Production*, **11**, 533-536, 2003.