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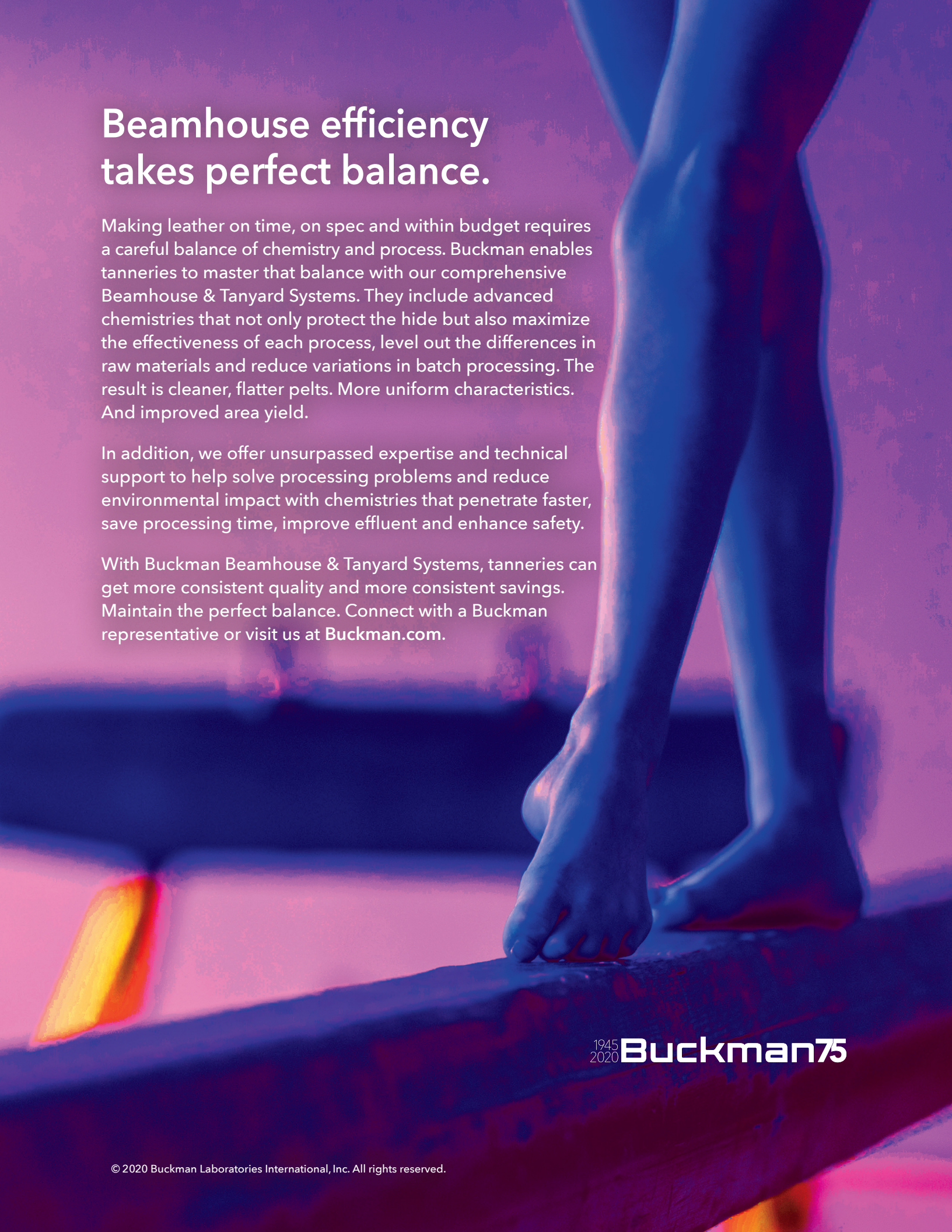

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Construction of a Chrome-free Tanning System Based on Highly-oxidized Starch–Zirconium Complexes

by

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Abstract

Chrome-free leather manufacture is one of the major focuses of leather industry. In this work, a chrome-free tanning system based on highly-oxidized starch–zirconium complexes (HOS–Zr) was constructed. Particle size and charge properties analyses revealed that the small size (2.5 nm) and the low cationic species content (45.1%) of HOS–Zr at low pH facilitated its uniform penetration in leather at the initial stage of tanning, and the large size (89–169 nm) and the high cationic species content (77.5%) of HOS–Zr after basification contributed to its stable crosslinking between collagen fibers. A 3% HOS–Zr offer (based on the weight of ZrO_2) imparted wet white with sufficient tanning effect. The physical properties of HOS–Zr crust leather were remarkably better than those of lactic acid–zirconium crust leather and were comparable to those of chrome crust leather. Wastewater in HOS–Zr tanning system showed a reduction in TOC load by 40.0% and higher biodegradability compared with chrome tanning system. As a result, this work provided a practical and sustainable approach to eliminate chrome in leather manufacture.

Introduction

Leather industry is facing an enormous challenge associated with the generation of chrome-containing wastewater and solid wastes that may pose a potential environmental risk.[1-3] Thus, green and sustainable chrome-free tanning agents and tannages should be developed to eliminate chrome from the source.[4-6] Owing to the good tanning performance, zirconium salt is regarded as a promising alternative to chrome tanning agent.[7] However, zirconium salt is easy to undergo hydrolysis and olation to form macromolecular cationic complexes even under low pH conditions,[8] thereby causing deposition on leather surface and uneven distribution in leather. Consequently, leather tanned with zirconium salt usually shows undesirable organoleptic properties.

Introduction of masking agents (ligands) is a common strategy to promote the penetration of zirconium salt in leather. The reported ligands can be divided into two categories, i.e., small molecular ligands such as lactic acid, citric acid, and tartaric acid,[9] and synthetic macromolecule ligands such as multi-carboxyl polymer and sulfonated tetraphenyl calix[4] resorcinarene.[10,11] Small molecular ligands are effective in strengthening the penetration of Zr complexes, since they can coordinate with zirconium salt and alleviate its surface binding. However, the complexes may be too small to form macromolecule crosslinking networks among collagen fibers.[12] Macromolecule ligands, which have abundant functional groups and wide molecular weight distribution, can improve the tanning performance of complex tanning agents. Nevertheless, these polymer ligands mainly used non-renewable petrochemicals as raw materials. Hence, designing a sustainable ligand is necessary for the development of chrome-free tannage.

We previously developed a type of environment-friendly highly-oxidized starch (HOS) ligand by using H_2O_2 and Cu–Fe catalyst.[13] The HOS ligand with proper carboxyl content and relatively large molecular size can coordinate with zirconium salt (HOS–Zr) and result in excellent tanning performance. Then research on the modified polysaccharides as ligand of non-chrome metal salts has been an emerging trend.[14-18] In this study, to further build a satisfactory chrome-free eco-tanning system, the tanning conditions of HOS–Zr were optimized in terms of the shrinkage temperature (T_s) of wet white, and the uptake rate of zirconium and HOS by leather. Then, the particle size and the charge properties of HOS–Zr in tanning liquor were analyzed to reveal its coordination behavior. Finally, HOS–Zr tanning system was systematically compared with conventional lactic acid–zirconium (Lac–Zr) and chrome tanning systems through tanning performance and environmental benefits analyses. This work aims to develop a practical and green chrome-free tanning system for the sustainable leather industry.

Experimental

Materials

Pickled cattle hide for the production of sofa leather was obtained from a local tannery. HOS with carboxyl group of 10.0 mmol/g and *Mw* of 6.8 kDa was prepared using starch, hydrogen peroxide, and a Cu–Fe catalyst, as described in our previous work.[13] Lactic acid (Lac), $Zr(SO_4)_2{\cdot}4H_2O$, hydrochloric acid, and sodium hydroxide were of

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analytical grade and supplied by Chengdu Kelong Chemical Co., Ltd. (Sichuan, China). Basic chromium sulfate (basicity 33%, Cr_2O_3 24%) was of industrial grade and supplied by Minfeng Chemical Co., Ltd. (Chongqing, China). The other reagents used for analysis were of analytical grade. The chemicals used for leather processing were of industrial grade.

Optimization of HOS–Zr Tanning System

Pieces of pickled cattle hides (60 cm × 30 cm) were repickled with 80% water (based on weight of limed hide), 7% sodium chloride, and 0.1% sulfuric acid at 25°C for 60 min. Then they were tanned with $Zr(SO_4)_2{\cdot}4H_2O$ (1%, 2%, 3%, and 4%, based on the weight of ZrO_2) and HOS (the mass ratio of HOS to ZrO_2 was 0.4:1, 0.8:1, 1.2:1) at 25°C for 1, 2, 3, and 4 h, respectively. This duration of tanning before basification was defined as a penetration stage. After the pH of tanning float was basified to 4.0 with magnesium oxide and sodium bicarbonate, 120% hot water (60°C) was added. The drums ran for another 2 h at 40°C, and then stayed overnight. The Zr and total organic carbon (TOC) concentrations of tanning floats before and after processing were measured by inductively coupled plasma optical emission spectroscopy (ICP-OES, Optima 8000, PerkinElmer, USA) and TOC analyzer (vario TOC, Elementar, Germany), respectively, and the uptake rates of Zr and HOS were calculated.[19] The T_s of the wet white leather was measured using a T_s tester (MSW-YD4, Sunshine Electronic Research Institute, China).

Particle Size Analysis

Particle size measurements of HOS solution (48 g/L) and HOS–Zr solution (containing 48 g/L HOS and 173 g/L $Zr(SO_4)_2{\cdot}4H_2O$) were performed at 25°C using particle size analyzer (Omni, Brookhaven, USA). The pH of the solution was adjusted to 0.5 and 3.0 by using HCl solution (1 mol/L) or NaOH solution (1 mol/L) before determination.

Charge Properties Analysis

The contents of anionic, electroneutral, and cationic species in the Zr (3.6 g/L $Zr(SO_4)_2{\cdot}4H_2O$), Lac–Zr (containing 1.0 g/L lactic acid and 3.6 g/L $Zr(SO_4)_2{\cdot}4H_2O$), and HOS–Zr (containing 1.0 g/L HOS and 3.6 g/L $Zr(SO_4)_2{\cdot}4H_2O$) complexes solutions were determined using a precipitation method.[18,20] One mL of Anionic precipitant (sodium diisobutyl naphthalenesulfonate, Xinrunde Chemical Co. Ltd., Hubei, China) was mixed with 20 mL complexes solution whose pH had been adjusted to 0.5 and 3.0 by using HCl solution (1 mol/L) or NaOH solution (1 mol/L). After centrifuging at 5000 r/min for 5 min, the precipitate was transferred and digested with nitric acid and hydrogen peroxide. The zirconium content in the digestion solution was detected by using ICP-OES and was defined as anionic species content. Likewise, 1 mL of cationic precipitant (a type of cationic polyamine resin, Jogel Industrial Co., Ltd., Shanghai, China) was mixed with 20 mL complexes solution (pH 0.5 and 3.0). Centrifugation, digestion, and ICP-OES detection were performed to obtain the cationic species content. The total zirconium content in complexes solution was determined by using ICP-OES after digestion. The percentage of anionic and cationic complexes were calculated as formulas (1) and (2), and the rest were defined as the percentage of electroneutral complexes.

$$\text{Anionic complexes percentage \%} = \frac{\text{Anionic species content}}{\text{Total zirconium content}} \times 100 \quad (1)$$

$$\text{Cationic complexes percentage \%} = \frac{\text{Cationic species content}}{\text{Total zirconium content}} \times 100 \quad (2)$$

Tanning Trials

Three pieces of pickled cattle hide (8 kg for each) were used for the tanning trials. The first piece was tanned with $Zr(SO_4)_2{\cdot}4H_2O$ (3 wt% ZrO_2, based on the weight of limed hide, the same below) and 2.4 wt% Lac ligand to obtain the Lac–Zr tanned leather (Table I). The second piece was tanned with the same dosages of $Zr(SO_4)_2{\cdot}4\ H_2O$ and HOS to obtain the HOS–Zr tanned leather (Table I). The last piece was tanned with 7% chrome tanning agent (1.68 wt% Cr_2O_3) to obtain the chrome tanned leather (Table II). After tanning, the T_s of the tanned leathers was measured using a T_s tester. The samples of tanned leather were split into three uniform layers by using a freezing microtome (CM1950, Leica, Germany), and then dried to constant weight at 102°C. The dried samples were digested, and their Zr/Cr contents were determined by using ICP-OES to calculate the ZrO_2/Cr_2O_3 contents (based on the weight of dry leather) in each layer of the tanned leathers. The samples of tanned leathers were cut into pieces (2 cm × 2 cm), dried at 45°C, and ground into particles (diameter less than 2 mm) by using a cutting mill (SM 100, Retsch, Germany), and then their isoelectric points (pI) were determined by using a zeta potential analyzer (MütekTM SZP-10, BTG, Germany).[21] The samples of tanned leather were lyophilized by using a freeze dryer (LGJ-30F, Xinyi, China), and the grain surface was observed by using a stereo microscope (M205 C, Leica, Germany). The distribution of Zr/Cr on the cross sections of leather was observed by using an energy dispersive x-ray spectroscopy (EDS, INCA X-MAX 50, Oxford, UK), and the cross section of leather was observed by using a scanning electron microscope (SEM, Pro X, Phenom, Netherlands).

The tanned leathers were wrung and shaved to uniform thickness of 1 mm, and then a typical post-tanning process (Table III) was conducted to obtain crust leathers. The crust leathers were conditioned at 20°C and 65% relative humidity for 24 h, and then their physical properties, such as softness, tensile strength, tear strength, bursting strength, elongation at break, compression performance, and resilience performance, were determined.[22] Finally, the grain surface was observed by stereo microscope and the cross section was observed by SEM.

Wastewater samples were collected at the end of each tanning and post-tanning process, and the volumes were measured. The Zr/Cr and TOC concentrations in wastewaters were analyzed as mentioned above. The Zr/Cr and TOC loads were calculated and expressed as kg/ton of pickled cattle hide. In addition, biochemical oxygen demand (BOD_5) and chemical oxygen demand (COD_{Cr}) of the wastewater samples were determined.[23,24]

Table I

La–Zr and HOS–Zr tanning process

Process	Material	Percentage (%)[a]	Temperature (°C)	Time (min)	Remarks
Repickling	Water	80	25		
	Sodium chloride	7			
	Sulfuric acid	0.1		60	
Tanning	$Zr(SO_4)_2{\cdot}4H_2O$	8.7	25		3% ZrO_2
	Lac /HOS	2.8		180	
	Magnesium oxide	1.2	25	30 × 4	
	Sodium bicarbonate	2.4		5 × 20	pH 4.0
	Water	120	40	120	Overnight, drain
Horse up for 24 h →Wet white					

[a] the percentage of material was based on the weight of limed hide.

Table II

Conventional chrome tanning process

Process	Material	Percentage (%)[a]	Temperature (°C)	Time (min)	Remarks
Repickling	Water	8	25		
	Sodium chloride	7			
	Sulfuric acid	0.1		60	
Tanning	Basic chromium sulfate	7	25	240	
	Sodium formate	1	25	30	
	Sodium bicarbonate	2		6 × 20	pH 4.0
	Water	120	40	120	Overnight, drain
Horse up for 24 h →Wet blue					

[a] the percentage of material was based on the weight of limed hide.

Table III

Post-tanning process

Process	Material	Percentage (%)[a]	Temperature (°C)	Time (min)	Remarks
Rewetting	Water	400	35		
	Degreasing agent	0.5		40	Drain
Washing	Water	400 × 2	35	10 × 2	Drain
Neutralizing	Water	200	35		
	Neutralization syntan	2			
	Sodium formate	1		30	
	Sodium bicarbonate	0.6 × 2		15 + 60	Drain, pH 6.0
Washing	Water	400 × 2	35	10 × 2	Drain
Retanning	Water	100	35		
	Acrylic resin	3			
	Dispersing syntan	1		30	
	Melamine resin	1			
	Dicyandiamide resin	2			
	Dyestuff	2		30	
	Dispersing syntan	1			
	Mimosa	5		60	
	Formic acid	0.4 × 2		10 + 30	Drain, pH 4.0
Fatliquoring	Water	150	50		
	Phospholipid	5			
	Sulfonated fatliquor	1			
	Synthetic fatliquor	9			
	Dyestuff	0.3		60	
	Formic acid	0.5 × 2		15 × 2	Drain, pH 3.8
Washing	Water	200	25	15	Drain
Horse up overnight →Hang drying → Conditioning → Milling → Crust leathers					

[a] the percentage of material was based on the weight of limed hide.

Results and Discussion

Optimization of HOS–Zr Tanning System

The tanning conditions of HOS–Zr, including mass ratio of HOS to ZrO_2, HOS–Zr dosage, and penetration time, were optimized to construct a satisfactory chrome-free tanning system. Figures 1 (a) and 1(b) show the effect of mass ratio of HOS to ZrO_2 on HOS–Zr tanning performance. The T_s of wet white reached a peak (89.6°C) when the mass ratio of HOS to ZrO_2 was 0.8:1. Further increase in the mass ratio resulted in a decline of the T_s of wet white because excess HOS with abundant carboxyl groups masked the Zr ion and blocked the binding sites between HOS–Zr and leather collagen fibers. This fact can also be verified by the decrease in the uptake rate of zirconium and HOS. Figures 1 (c) and 1(d) show the effect of HOS–Zr dosage on its tanning performances. High dosage resulted in high T_s of wet white but low uptake rate of zirconium and HOS because of the limited coordination sites on the leather collagen fibers. The T_s of wet white tanned with 3% HOS–Zr was 88.1°C, which met the common requirement for storage, transportation and trade in the leather industry.[19] As shown in Figures 1 (e) and 1(f), prolonging the penetration favored the uptake of HOS–Zr in leather, and thus enhanced the T_s of wet white. When the penetration time was higher than 3 h, the T_s of wet white and the uptake of HOS–Zr reached a plateau. Thus, the optimized HOS–Zr tanning conditions was summarized as follows. The mass ratio of HOS to ZrO_2 was 0.8:1. The HOS–Zr dosage was 3%. The penetration time was 3 h.

Particle Size of HOS–Zr

Leather collagen fibers possess a hierarchical structure assembled by microfibrils, fibrils, fibers and fiber bundles,[25] and its pore size distribution ranges from 7 Å to 150 μm.[26] Theoretically, the tanning agents with too large molecular size may be difficult to penetrate into the microfibrils and fibrils (Φ 50-200 nm), whereas those with too small molecular size may be hard to form effective crosslinking bond between fibers (Φ 2-10μm). Thus, a proper size is an essential factor for a tanning agent to show excellent tanning performance. Here, we performed particle size analysis to verify whether HOS–Zr is suitable for tanning. The particle size distribution of HOS and HOS–Zr solutions at different pH values are shown in Figure 2. When the pH was 0.5, HOS showed two peaks around 1.0 μm and

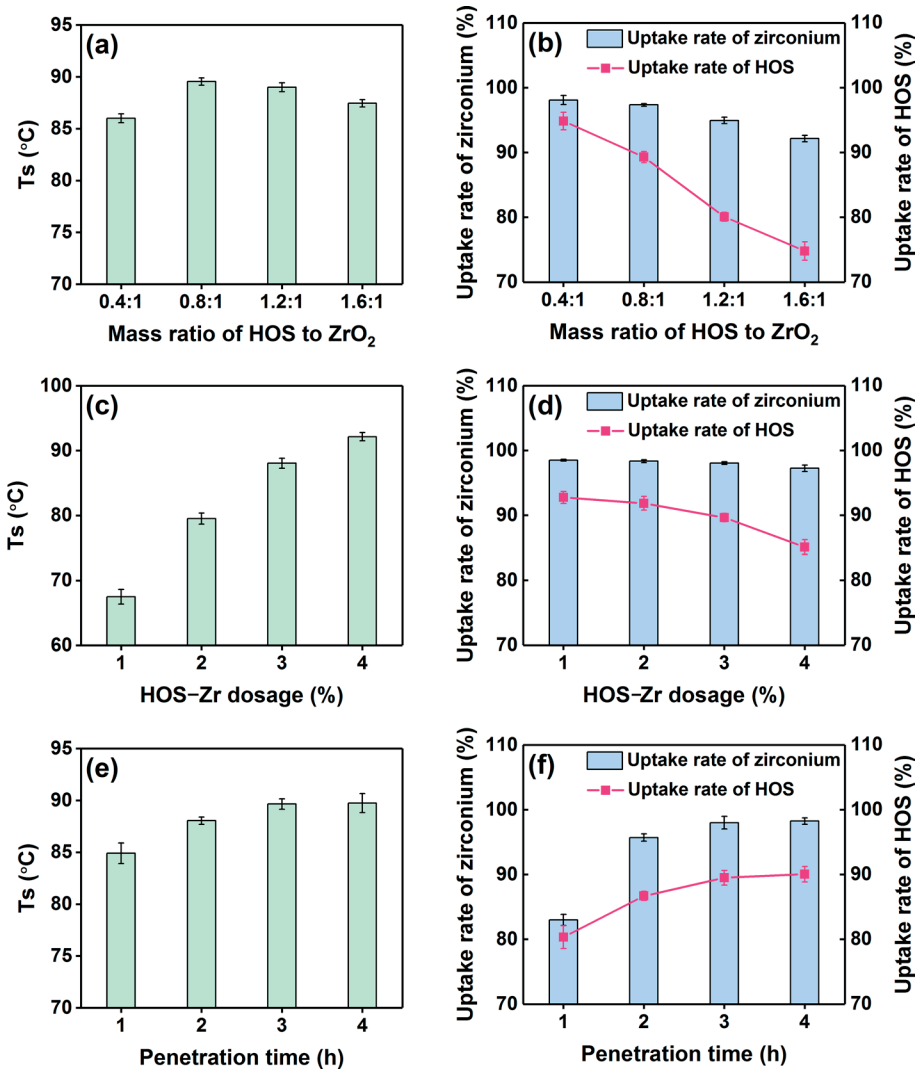

Figure 1. Effects of mass ratio of HOS to ZrO2 **(a, b)** (HOS–Zr dosage 3%, penetration time 3 h), HOS–Zr dosage **(c, d)** (mass ratio of HOS to ZrO2 0.8:1, penetration time 3 h), penetration time **(e, f)** (mass ratio of HOS to ZrO2 0.8:1, HOS–Zr dosage 3%) on Ts of wet white and uptake rate of zirconium and HOS by leather.

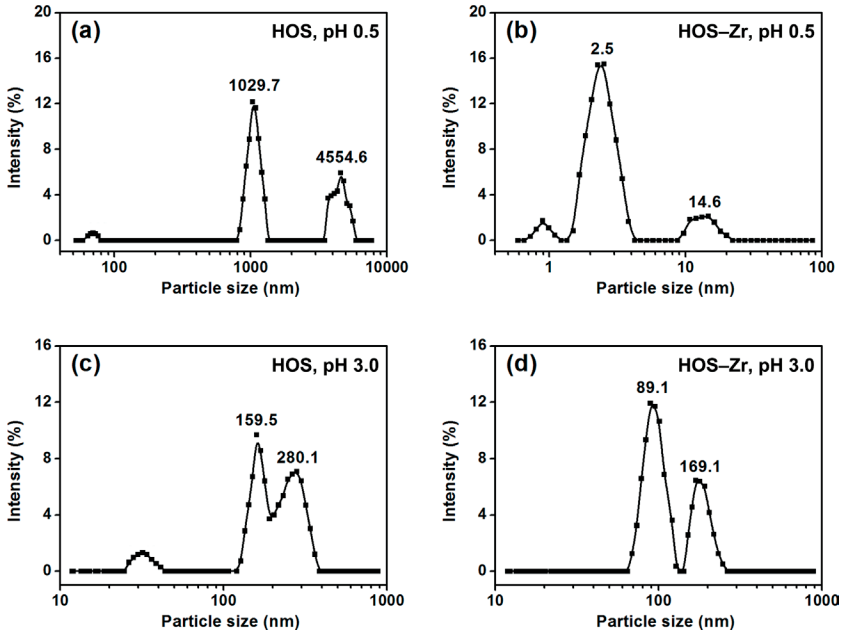

Figure 2. Particle size distribution of HOS and HOS–Zr at pH 0.5 **(a, b)** and at pH 3.0 **(c, d)**.

4.5 μm (Figure 2a), indicating the formation of serious aggregation structures. However, HOS–Zr exhibited a prominent peak around 2.5 nm (Figure 2b) because the formation of HOS–Zr complexes weakened the hydrogen bonding and van der Waals forces among HOS molecules.[27] The small size of HOS–Zr ensured its penetration in the hierarchical structure of collagen fibers at the beginning of tanning (pH 0.5). As the pH increased to 3.0, the partial deprotonation of carboxyl group made the particle size of HOS decreased to 159.5 nm and 280.1 nm (Figure 2c). The hydrolysis and olation of HOS–Zr made its particle size increase to 89.1 nm and 169.1 nm (Figure 2d), which favored its crosslinking among collagen fibers after basification (pH > 3.0). Therefore, HOS–Zr showed a good potential in balancing its penetration and fixation in leather.

Charge Properties of Zr Complexes

The penetration and fixation of a tanning agent in leather is also influenced by its charge properties in tanning float.[18] According to the theory of tanning chemistry, a mineral tanning agent is mainly coordinated with carboxyl anions of collagen through its cationic species. A tanning agent with low cationic species content is easy to penetrate into leather, while that with high cationic species content is prone to bind with collagen.[28] Based on this theory, we investigated the charge properties of Zr, Lac–Zr, and HOS–Zr complexes at different pH values, and the results are shown in Figure 3. When the pH was 0.5, the cationic species content in complexes followed the order as HOS–Zr (45.1%) < Lac–Zr (51.7%) < Zr (56.7%). This result indicated that HOS–Zr will be easier to penetrate into the leather

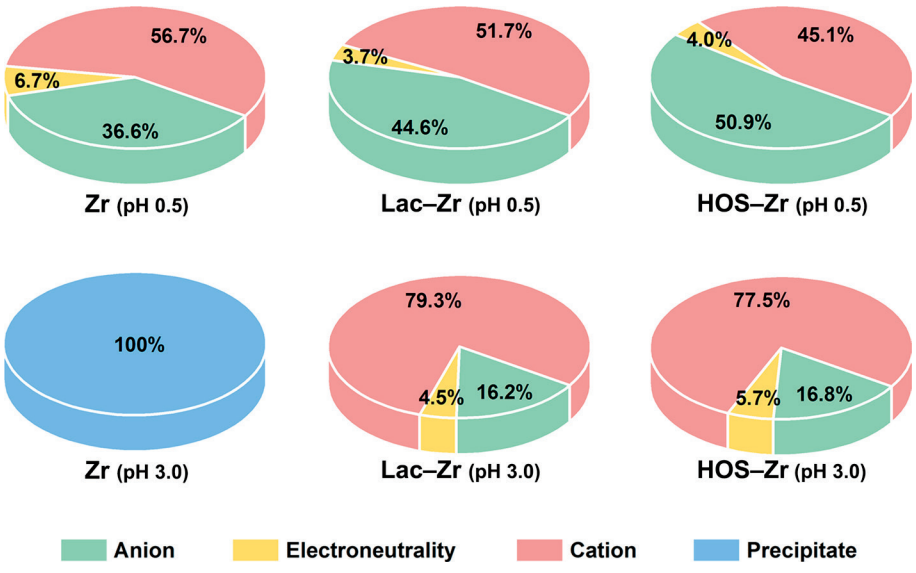

Figure 3. Charge compositions of Zr, Lac–Zr, and HOS–Zr complexes at pH 0.5 and pH 3.0.

in the early stage of tanning (pH 0.5) than Zr and Lac–Zr, which should be attributed to the fact that the introduction of HOS ligand masked the Zr ion more effectively and reduced the electropositivity and reactivity of Zr complexes. When the pH rose to 3.0, pure Zr salt completely precipitated, showing weak stability to alkali. Lac–Zr and HOS–Zr were still in aqueous solution. The cationic species content of HOS–Zr (77.5%) was close to that of Lac–Zr (79.3%), indicating that they had similar binding capacity with collagen fibers after basification (pH > 3.0). Based on the results above, HOS–Zr is expected to have better tanning performance than Lac–Zr and Zr.

Comprehensive Evaluation of the Tanning Performance of HOS–Zr

The tanning performance of HOS–Zr was systematically compared with that of Lac–Zr and chrome to demonstrate its practicability. As shown in Table IV, ZrO_2 distribution uniformity of HOS–Zr tanned leather was obviously higher than that of Lac–Zr tanned leather, which could also be demonstrated by EDS analysis (Figure 4). This result confirmed the speculation of the last section that HOS–Zr complexes can uniformly penetrate and distribute in the leather matrix because of the masking effect of HOS. Accordingly, the T_s (Table IV), the grain surface morphology, and the fiber dispersion degree (Figure 5) of HOS–Zr tanned leather were substantially better than those of Lac–Zr tanned leather, even close to those of chrome tanned leather. The pI of leather plays an important role in leather processing, since it greatly influences the penetration and the fixation of post-tanning chemicals in leather matrix.[21] The pI of HOS–Zr tanned leather (7.03, see Table IV) was close to that of chrome tanned leather (7.13), implying an excellent compatibility of the HOS–Zr tanned leather to the existing post-tanning process that was designed for chrome tanned leather.

The properties of HOS–Zr crust leather were further evaluated. Table V shows that all the physical properties of the HOS–Zr crust leather were better than those of Lac–Zr crust leather because of the favorable distribution and fixation of HOS–Zr in leather. Compared with chrome crust leather, HOS–Zr crust leather exhibited higher tensile strength, tear strength, and bursting strength, but lower softness, elongation, compression and resilience performance (Figure 6), probably because of the strengthening effect and strong filling ability of HOS–Zr on collagen fibers. Figure 7 shows that HOS–Zr crust leather presented a finer grain and a looser fiber network than Lac–Zr crust leather, which was similar with those of chrome crust leathers. Therefore, HOS–Zr tanning agent is qualified for leather manufacture because of the reliable physical properties of the resultant leather.

Table IV

Properties of tanned leathers

	ZrO_2/Cr_2O_3 (based on dry weight of leather, %)				
Sample	**Grain**	**Middle**	**Flesh**	**Ts (°C)**	**pI**
Lac–Zr	8.41 ± 0.03	7.79 ± 0.06	8.32 ± 0.04	81.4 ± 1.3	6.27
HOS–Zr	8.99 ± 0.06	8.67 ± 0.08	8.81 ± 0.03	91.0 ± 0.6	7.03
Chrome	4.91 ± 0.04	4.68 ± 0.03	4.71 ± 0.04	109.9 ± 1.3	7.13

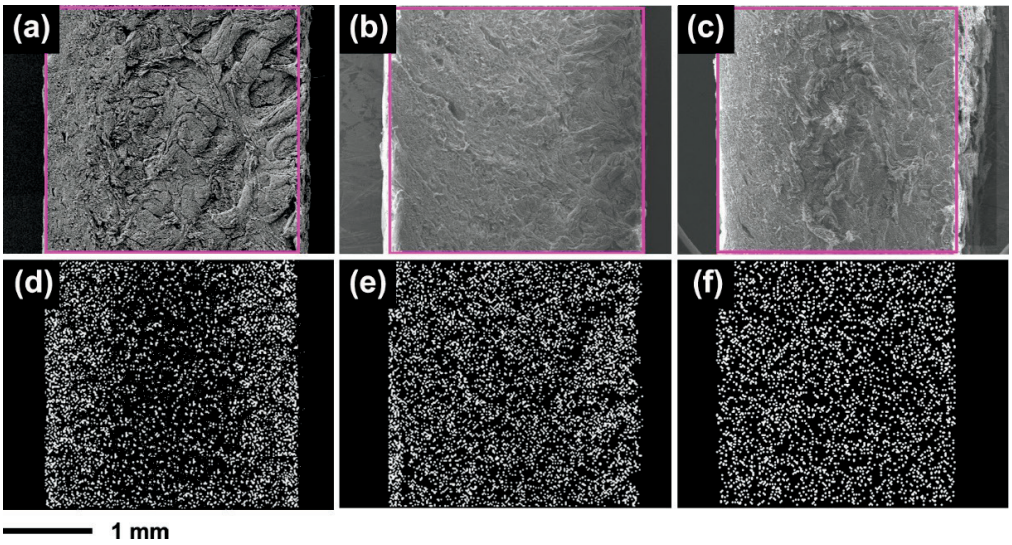

Figure 4. SEM images of cross section of Lac–Zr **(a)**, HOS–Zr **(b)**, and chrome **(c)** tanned leathers. EDS images of the distribution of zirconium/chromium in cross section of Lac–Zr **(d)**, HOS–Zr **(e)**, and chrome **(f)** tanned leathers.

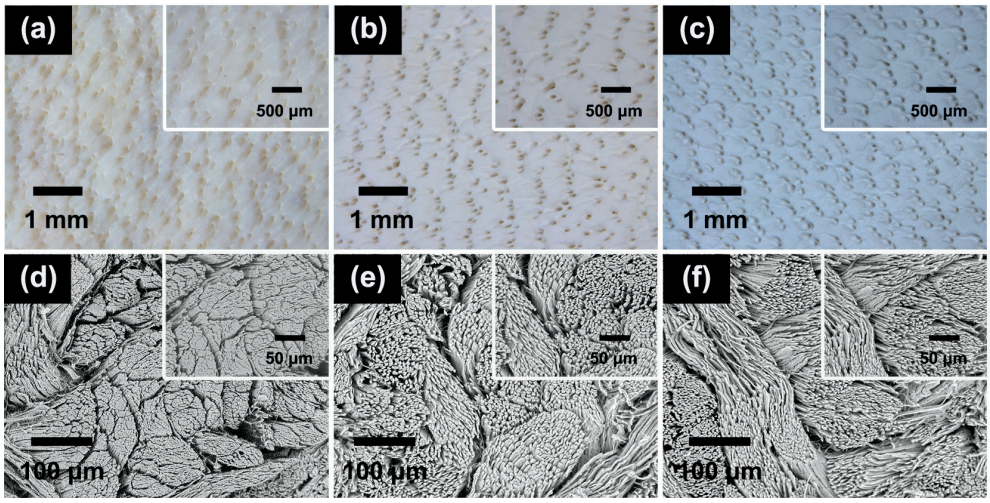

Figure 5. Stereo microscope images of grain surface of Lac–Zr **(a)**, HOS–Zr **(b)**, and chrome **(c)** tanned leathers. SEM images of cross section of Lac–Zr **(d)**, HOS–Zr **(e)**, and chrome **(f)** tanned leathers.

Table V
Physical properties of crust leathers

Crust leather sample	Softness(mm)	Tensile strength (N/mm^2)	Tear strength (N/mm)	Bursting strength (N/mm)	Elongation at break (%)
Lac–Zr	7.0 ± 0.2	10.6 ± 1.1	34.3 ± 2.6	275.6 ± 21.5	50.7 ± 3.9
HOS–Zr	8.3 ± 0.1	14.9 ± 1.0	49.8 ± 1.8	342.5 ± 9.7	46.1 ± 4.9
Chrome	8.4 ± 0.1	13.7 ± 0.9	44.8 ± 3.2	289.7 ± 15.2	55.9 ± 6.5

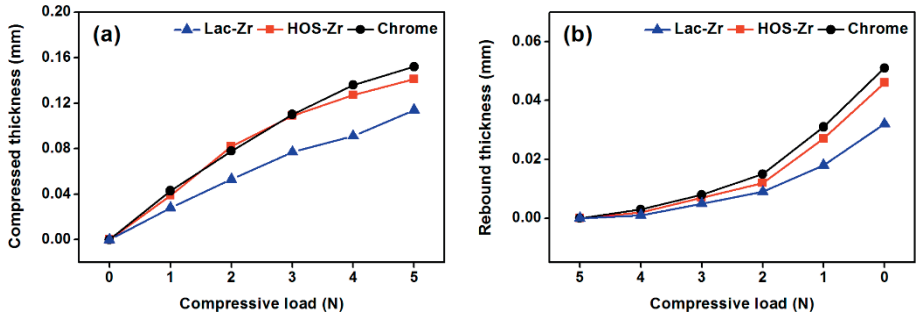

Figure 6. Compression **(a)** and resilience **(b)** performance of crust leathers.

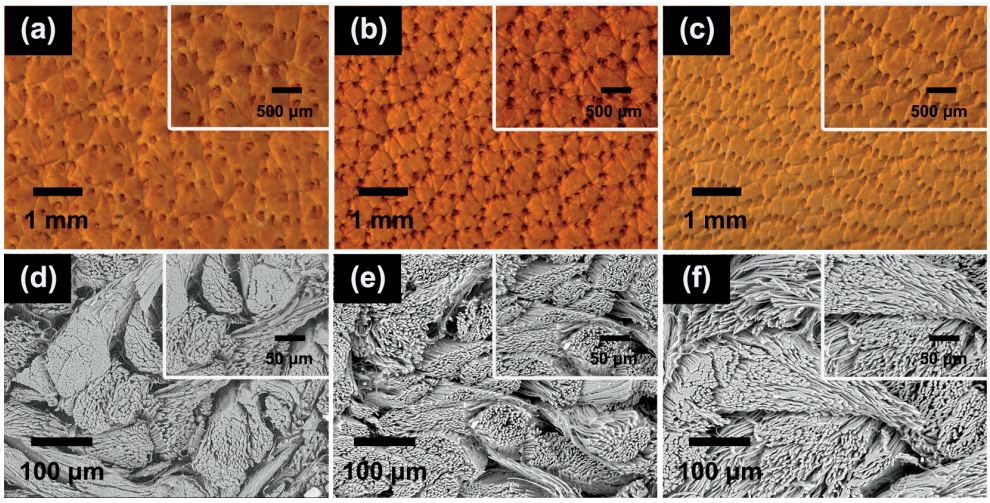

Figure 7. Stereo microscope images of grain surface of Lac–Zr **(a)**, HOS–Zr **(b)**, and chrome **(c)** crust leathers. SEM images of cross section of Lac–Zr **(d)**, HOS–Zr **(e)**, and chrome **(f)** crust leathers.

Environmental Impact Assessment

Table VI shows the Zr/Cr and TOC loads in tanning and post-tanning wastewaters. Lac–Zr and HOS–Zr tanning systems did not produce chrome-containing wastewater since no Cr was introduced. Chrome output in conventional chrome tanning system was 7.73 kg/ton of pickled hide. The Zr load in HOS–Zr wastewater (0.29 kg/ton of pickled hide) was dramatically lower than that in Lac–Zr (0.88 kg/ton of pickled hide) wastewater, implying that HOS–Zr was stably fixed on collagen fibers. As for the discharge of organics, the TOC load in HOS–Zr wastewater were reduced by 54.2% and 40.0%, respectively, compared with that in Lac–Zr and chrome wastewaters. The reduction in TOC load was mainly concentrated in retanning and fatliquoring wastewaters, suggesting that HOS–Zr tanned leather exhibited higher uptake for anionic post-tanning chemicals than Lac–Zr and chrome tanned leathers. This finding should be ascribed to the strong electropositivity of HOS–Zr tanned leather, which is consistent with results of our previous study.[21] Thus, considerable environmental benefits can be achieved by using HOS–Zr tanning system in terms of the reduction in pollution loads.

The ratio of BOD_5/COD_{Cr} is usually used for evaluating the biodegradability of wastewater. A higher ratio means a better biodegradability.[29] Table VII shows the biodegradability of tanning and post-tanning wastewaters for the three tanning systems. HOS–Zr tanning wastewater was more biodegradable than Lac–Zr and chrome tanning wastewaters because the ligand, HOS, was originated from natural polysaccharide. Additionally, the BOD_5/COD_{Cr} ratio of HOS–Zr post-tanning wastewater (0.37) was higher than that of Lac–Zr (0.30) and chrome post-tanning wastewaters (0.24). The reason may be that the low metal ion and organic contents in HOS–Zr post-tanning wastewater were conducive to microorganism growth. Thus, the HOS–Zr wastewater with high biodegradability should be convenient to treat.

Conclusions

A chrome-free eco-tanning system for eliminating chrome discharge has been constructed based on HOS–Zr complexes. The introduction of HOS greatly reduced the electropositivity and reactivity of the Zr complexes, thereby resulting in uniform distribution and effective crosslinking of the complexes in leather. The tanning performance of HOS–Zr was comparable to those of the chrome and was remarkably better than those of Lac–Zr. The HOS–Zr tanning system completely eliminated chrome discharge and reduced TOC load in tanning and post-tanning wastewater by 40% compared with chrome tanning system. Moreover, HOS–Zr wastewater showed preferable biodegradability. Thus, HOS–Zr tanning system is promising to be applied in tanneries due to its reliable leather quality and the environmental benefits.

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Table VI

Pollution loads in tanning and post-tanning wastewaters (unit: kg/ton of pickled hide)

	Zirconium output		Chromium output	TOC output		
Process	Lac–Zr	HOS–Zr	Chrome	Lac–Zr	HOS–Zr	Chrome
Tanning	0.51 ± 0.01	0.15 ± 0.00	7.34 ± 0.01	10.23 ± 0.11	8.49 ± 0.05	6.10 ± 0.11
Rewetting*	0.13 ± 0.00	0.07 ± 0.00	0.28 ± 0.01	3.16 ± 0.05	3.02 ± 0.05	1.87 ± 0.01
Neutralizing*	0.14 ± 0.00	0.04 ± 0.00	0.02 ± 0.00	4.52 ± 0.12	3.21 ± 0.18	2.74 ± 0.05
Retanning	0.03 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	9.56 ± 0.12	5.36 ± 0.08	9.49 ± 0.08
Fatliquoring*	0.07 ± 0.00	0.02 ± 0.00	0.08 ± 0.00	52.00 ± 0.55	16.38 ± 0.42	40.55 ± 0.47
Total	0.88 ± 0.02	0.29 ± 0.01	7.73 ± 0.03	79.47 ± 0.93	36.46 ± 0.78	60.75 ±0.71

*Including the following washing process.

Table VII

Biodegradability of tanning and post-tanning wastewaters

Wastewater sample	COD_{Cr} (g/L)		BOD_5 (g/L)		BOD_5/COD_{Cr}	
	Tanning	Post-tanning	Tanning	Post-tanning	Tanning	Post-tanning
Lac–Zr	3.21 ± 0.07	9.14 ± 0.15	0.75 ± 0.07	2.70 ± 0.21	0.23	0.30
HOS–Zr	2.62 ± 0.05	4.10 ± 0.06	0.85 ± 0.07	1.50 ± 0.14	0.32	0.37
Chrome	1.96 ± 0.04	7.17 ± 0.08	0.25 ± 0.07	1.70 ± 0.14	0.13	0.24

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A Novel Approach of Removing Externally Attached Debris from Animal Carcass to Ensure Meat Safety and Byproduct Quality

by
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Abstract

In this study, a formulation and technique are developed to be used for cattle carcass decontamination prior to removal of hide in a commercially preferred time-frame to ensure meat safety and byproduct quality. This formulation offers deep cleaning on carcass surface by removing debris including manure/mud balls which are firmly attached to the hair of animal hide harboring pathogens like *Salmonella* and *Escherichia coli*. Survival of such pathogens can facilitate cross-contamination of the underlying meat and meat-processing equipment in the packing plant posing a challenge to the meat industry as well as public-health. Also, the attached adobe type mud/manure balls have potential to create holes on the hide during leather processing which degrades byproduct's quality. Formulation was sprayed on cattle's hide and the attached debris were brushed off from the surface. The formulation was found very efficient in cleaning the hide surface both at 5 and 8 min treatments. The highest of aerobic, *Escherichia coli* and *Salmonella* populations were reduced by 8.71, 3.63 and 3.19 Log CFU/50 in^2, respectively when compared to water-wash. The efficacy of formulation can be optimized by adjusting its concentration and treatment time. Post-leather analysis showed no detrimental impact on byproduct caused by the formulation.

Introduction

Animal meat and hides are the main product and byproduct, respectively of the meat industries. In the meat processing facility (e.g., beef and pork), animals undergo a process where they are stunned, bled, flayed, eviscerated, and assembled into small pieces of meat that are packaged for public consumption or restaurant trade. Separately, after skinning, hides are processed into leather, a valuable commodity. Prior to entering the meat processing facility, animals are externally tarnished with various foreign materials such as dirt, manure, mud, and plant materials that adhere and entangle on their hair as well as microbial contamination. These debris, in particular hardened manure and mud balls (e.g., adobe-type), not only hinder the proper cleaning process[1-5] of the animal surface posing the threat of microbial cross-contamination from hide to underlying meat during skinning but also often cause damage such as holes in the hide during leather processing and destroy this valuable byproduct of the meat industry.

Research studies[6-10] showed, usually the interior portion of a carcass containing the meat is sterile, however bacterial contamination occurs because of transfer from hide/skin onto the meat during the slaughter and the hide/skin removal processes. In many cases, this bacterial contamination contains microorganisms that are pathogenic to humans. Enteric pathogenic bacteria, for example, on cattle surfaces serve as significant hazard and pose a substantial challenge to the meat industry as well as to public health. Such pathogens may arise from environmental exposures including from soil and manure during the lifespan of cattle, which may become firmly lodged onto their hides and hair and thus limits cleaning and decontamination efficacy. Therefore, it is important to properly clean the carcass through the removal of external debris before opening up the hide/skin.

Incomplete decontamination of carcasses prior to hide removal serves as a prime source of pathogen transfer to meat during slaughterhouse processing that leads to numerous public health risks and substantial economic loss. Previous studies revealed that meat contamination with pathogens is strongly correlated to hide contamination.[11-12] Due to the pathogenic contamination, a handful number of meat products of different meat processing plants have been recalled in recent years. For instance, an outbreak of *Salmonella* Dublin was linked to ground beef which caused a recall of 35k pounds of ground beef. In this incident, thirteen people were infected from eight states, where nine individuals were hospitalized including one death reported from California.[13] Another outbreak in 2018 caused a huge recall of a total 12.1 million pounds of beef products that was contaminated with *Salmonella* Newport reporting 333 cases in 28 states causing 99 individuals to be hospitalized.[14] In 2013, *Salmonella* Typhimurium was linked to an outbreak in Arizona, Illinois, Iowa, Michigan, Pennsylvania, and Wisconsin, where, 22 illnesses were reported, which was tracked back to two potential companies.[15] Although many *E. coli* are benign and are commonly found in the digestive tracts of mammals, some *E. coli* can cause major health issues, including diarrhea, urinary tract infections, respiratory illness, and bloodstream infections. An outbreak of *E. coli* infections linked to ground beef happened in 2018, where 18 cases were reported in four states with one death and six hospitalizations.[16] In

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Title: Removing of Manure/Mud Balls from Animal Carcass to Ensure Meat Safety and Byproduct Quality

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2016, veal, beef and cattle products contaminated with *E.coli* from an arbitrator in Massachusetts caused a multistate food related outbreak in Connecticut, Massachusetts, Pennsylvania, and West Virginia and a recall was enacted on meat products from the specific vendor.[17] In 2014, over 1.8 million pounds of ground beef from a packing facility in Michigan was recalled due to its association with an outbreak of cases in Massachusetts, Michigan, Missouri, and Ohio.[18] Investigations of such widespread outbreaks often conclud that, contamination likely occurred in meat processing facilities due to the transfer of pathogens to meat either from haired surface of animal or their environmental contaminants or processing equipment.

Accumulation of external debris on cattle/animal surface mainly happens during the cold season. The mechanism of adobe-type mud/manure ball formation, for example, is liken to a freeze-thaw process where the manure and/or mud are accumulated on the hide hair and hardens as the temperature approaches to freezing. As the cycle repeats, it creates hardened mud and/or manure that becomes exceedingly attached and entangled with the hide hair. If not removed, adobe-type mud/manure balls serve as safe harbor for the microorganism including pathogens and remain firmly attached to the hair when the hides are delivered to tanneries for leather processing. In the tannery, the mechanical (using fleshing machine) process of forcefully removing the mud balls from the hair causes damage to the hide by creating holes, which results in unusable hides/skin or poor-quality leather products.

There are a few methods that have been reported previously that had limited success to partially address this issue. For example, soaking the hides in solutions containing glycerol and sodium carbonate with/without surfactants, enzymatic formulations, and oxidative chemicals such as sodium percarbonate with or without an additional caustic agent.[19-20] First of all, these methods are time consuming in terms of industrial time management and more importantly those soaking methods can only deal with hide/skin not carcass therefore, not applicable in removing mud/manure from carcass prior to removal of hide/skin, thus offer no role in meat safety.

Debris on cattle surface including mud/manure balls serves as a prime source of microbial carcass contamination during animal slaughter and meat processing. Spray washing with water alone or with any washing formulation[21-25] which are currently standard in the industry, for example, have limited effectiveness to remove foreign materials and decontaminate the surface of the hide because washing solutions cannot reach under the debris. Therefore, it is important to remove mud/manure balls or any other external debris to decrease bacterial contamination on animal carcasses prior to hide removal to reduce the risk of human exposure to these microorganisms. Mechanical removal methods such as shaving is inefficient, cumbersome, and inadequate to fully remove the foreign materials and contamination.

There thus exists an ongoing industrial need to develop methods for efficiently cleaning foreign materials including mud/manure balls from animal hides as well as decontamination of animal carcasses. Under this study, a novel formulation and method have been developed for cleaning and decontaminating animal carcass prior to slaughter. More specifically, the invention relates to a complete protocol for removing foreign materials and microorganisms including pathogens from the surface of animal carcass. This will improve meat safety and prevent cross-contamination in meat packing facilities as well as to lower the likelihood of damage of hides delivered to tanners for leather processing.

Materials and Methods

Hide Preparation

De-fleshed bovine hides were collected from a local meat processing facility, JBS Packerland (Souderton, PA). For the experiments, the sections of the hide (from belly and butt areas) which contained most of the external debris including mud/manure balls were cut into pieces of approximately equal size of 12-inch × 12-inch.

Preparation of Decontamination Formulation

Aqueous solution (40-44%) of potassium thioglycolate (K-TG) was purchased from Across Organics. Sodium dichloroisocyanurate dehydrate (SDCC) and sodium hydroxide (NaOH) were purchased from Aldrich Chemical (Milwaukee, WI). Different decontamination formulations were prepared by dissolving/mixing the chemicals in tap water using the concentrations as described in Table I. All

Table I

Composition of carcass decontamination formulation

Formulations	Composition
F-A (control)	Tap water
F-B	2.5% NaOH (wt./v) + 2.5% K-TG (wt./v)
F-C	5% NaOH (wt./v) + 5% K-TG (wt./v)
F-D	7.5% NaOH (wt./v) + 7.5% K-TG (wt./v)
F-X	0.75% SDCC (wt./v)
F-BX	2.5% NaOH (wt./v) + 2.5% K-TG (wt./v) + 0.75% SDCC (wt./v)
F-CX	5% NaOH (wt./v) + 5% K-TG (wt./v) + 0.75% SDCC (wt./v)
F-DX	7.5% NaOH (wt./v) + 7.5% K-TG (wt./v) + 0.75% SDCC (wt./v)

concentrations were dissolved in tap water at room temperature (~22°C) and prepared ~24 h prior to the experimental spray applications on hides.

Spray Treatment on Bovine Hide Panels

Decontamination formulations and water (control) were dispensed from a hand-held 1 Lt. polyethylene spray bottle to the haired surface of individual hide panels containing firmly attached external debris. To cover the whole hide panel (approximately 12 in × 12 in surface area) adequately, a certain amount of 25 mL (25 puffs) of tap water and different formulations were sprayed on individual sample panels. The formulation was allowed to sit for 5 to 8 min before brushing to remove the debris and taking samples for microbial testing.

Manure/Mud Balls Removing Protocol

Picture of each hide panel (Figure 1) was taken before and after the mud/manure/debris removing experiment. After certain time of spray treatment, the hide pieces treated with water and formulation were brushed with a high heat resistant polymeric hand brush to wipe off the debris attached to the haired surface of the hide. Similar forces were applied to brush all the panels. In between the hide panels, the brush was disinfected dipping it in hot water to minimize cross-contamination.

Microbial Testing

After brushing the treated hide panels to get rid of debris, a 10 in × 5 in surface area was independently swabbed with a sterile sponge and placed into a corresponding sampling bag with 25ml of buffered peptone water for analysis (Nasco Meat and Turkey Carcass Sampling Kit, Salida, California). The sample bags were then hand massaged for ~2 min. Samples were serially diluted and spread-plated on Tryptic Soy Agar (TSA), Xylose-Lysine-Tergitol 4 (XLT-4) Agar, Sorbitol MacConkey Agar, with Cefixime and Tellurite (CT-SMAC) for aerobic bacteria, Salmonella and E. coli counts, respectively (all agar was obtained from Fisher Scientific, Pittsburg, PA). After spread plating, samples were incubated between 24 to 48 h at 37°C and bacterial colonies were enumerated for bacterial recovery with the lowest detection level at 1 CFU per 10 in × 5 in area.

Leather Processing and Quality Evaluation

After collecting the microbial samples, the treated hide panels were subjected to tanning to convert them into crust leather following the established USDA tanning protocol.[26-27] To evaluate the final impact of newly developed manure/mud/debris removing formulations on byproduct quality, each leather panel underwent a series of quality tests. These included organoleptic evaluation (break, handle, fullness, and color) and microscopic analysis (data is not included). In the

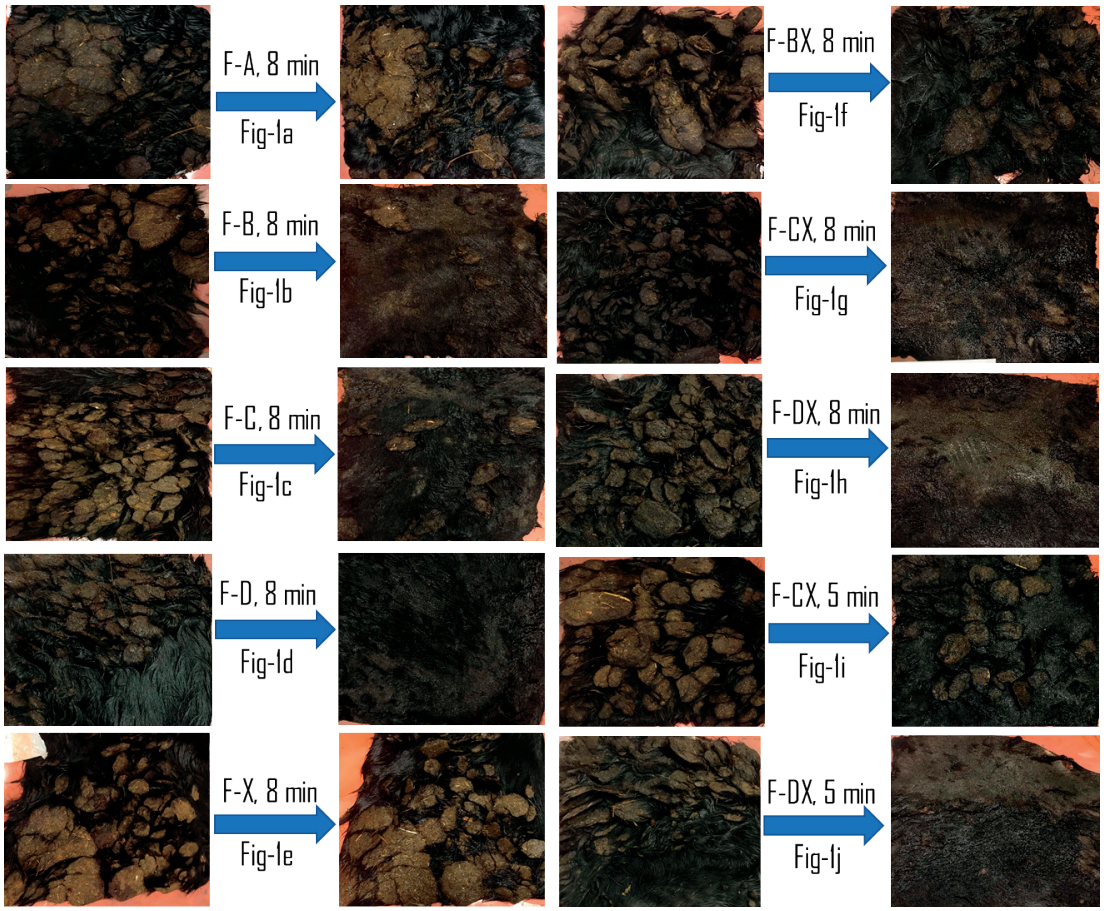

Figure 1. Efficacy of inventive formulations in removing debris from the haired surface of fresh bovine hide.

microscopic analysis, the leather samples were analyzed under a stereo microscope to determine any difference in the grain structures between the formulation treated samples (F-B, F-C, F-D, F-X, F-BX, F-CX, F-DX) and the control (F-A). The leather samples were also subjected to mechanical property analysis such as, tensile strength, elongation ("stretchability"), Young's Modulus ("stiffness") and fracture energy (energy required to fracture the leather sample). All the quality analyses of leather samples produced from the treated hide panels were carried out according to the published procedures.[2,21,28]

Statistical Analysis

All statistical analyses were carried out by using one-way analysis of variance (ANOVA) using Dunnett's comparison tests or unpaired t-tests. All calculations were carried out using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Significance was observed at $p < 0.05$.

Results and Discussion

Previous studies revealed that, meat contamination with pathogens is strongly correlated to hide contamination.[11-12] Therefore, it is important to remove the external debris from the cattle surface prior removal of hides to facilitate the proper cleaning of carcass. It offers not only better limit of surface microorganism including pathogens which pose threat to be migrated to the meat but also removing of debris ensures the quality assurance of byproduct.

As it is found that, those debris, especially adobe type manure/mud balls, are firmly attached to the hair therefore, in this investigation the developed formulation targets to weaken the hair to get off those debris. The inventive formulation combines two types of chemicals dissolved in aqueous solution, 1) manure/mud balls removing chemicals and 2) anti-microbial agent. Manure removing chemicals target to break down the hair by weakening the disulphide bonds of keratin. This formula consisted of a base such as sodium hydroxide (NaOH) and a salt of thioglycolic acid ($HSCH_2COOH$) for example potassium thioglycolate (K-TG). Thioglycolate dissolves the disulphide bonds in keratin where the base increases the pH which helps deliver thioglycolate in the hair. To strengthen anti-bacterial activity, an anti-microbial agent, *N*-halamine (R_1,R_2-N-X) was combined with manure removing chemicals to develop the final formulation. *N*-halamine represents a group of compounds with one or more nitrogen-halogen covalent bonds. They exhibit biocidal properties because of the +1 oxidation state of halide atoms in their molecule. *N*-halamine compounds are stable in aqueous solution and effective in limiting a broad spectrum of microorganism. They are cheap, weakly toxic, less corrosive than bleach, safe to humans and environmentally friendly.[29-32] For this research experiment, Sodium dichloroisocyanurate dehydrate (SDCC) was chosen because of its aqueous solubility. All the chemicals chosen for the formulation impose no/less toxicity as they are being used for human consumption in other applications.[33-34]

Efficacy of Formulation in Removing Debris from Hide Surface

As shown in Figure 1, the brushing after rinsing with water (control, Fig 1a) had no effect on removing debris from the hide surface. Similarly, treating with only SDCC (Fig 1e) solution resulted no removal of mud/manure balls. It was also shown that, the removal of debris including mud/manure balls depended both on the concentration and treatment time. At 8 min treatment, the highest concentration of formulation, F-D and F-DX (Fig 1d and Fig 1h) resulted in better performance in compare to F-B and F-C (Fig1b and Fig 1c) or F-BX and F-CX (Fig 1f and Fig 1g) by removing all the debris from hide surface. Also, F-C (Fig 1c) and F-CX (Fig 1g) worked better than F-B (Fig 1b) and F-BX (Fig 1f), respectively. In time variable experiments, 8 min treatment of F-CX (Fig 1g) worked better that the 5 min treatment (Fig 1i). However, the highest concentration of formulation F-DX showed equal level of efficiency in removing debris as it completely cleaned the hide surface both in 8 min (Fig 1h) and also in 5 min (Fig 1j). For such a short-time effectiveness, this formulation can be used in rapid industrial settings. Also, concentration and time variables can be adjusted based on the need of individual meat processing plants to establish an economically feasible setup.

Efficacy of Formulation in Reducing Bacterial Population from Hide Surface

Generally, underlying meat surface of carcass is sterile, but it can be contaminated as a result of pathogen transfer from hides onto the meat during slaughter and the hide removal process. Microbial decontamination of carcass prior to removal of hide is essential to minimize the risk of pathogen cross-contamination.

Aerobic Bacteria Colony Count

According to Table II, the treatment with every single formulation resulted in more reduction of aerobic bacteria comparing to the control. The formulations even without containing SDIC (F-B, F-C and F-D) also showed significantly better reduction than the control (F-A). This is because of removing debris which harbor microorganisms. It is also noteworthy to mention that, only antimicrobial solution (F-X) did not show the effectiveness in reducing aerobic bacteria as such as other formulations. This is due to the fact that, the formulation spray could not reach many places of the hide panel covered by the external debris to effectively kill the bacteria. Aerobic bacteria counts were reduced with the increased concentration of formulation meaning less debris on hide offers better decontamination. Formulation containing SDIC, F-BX, F-CX and F-DX resulted in 1.54, 2.32 and 3.28 Log CFU more reduction in compare to F-B, F-C and F-D, respectively. Comparing the treatments between 8 min and 5 min, results showed better reduction at longer time. Formulations F-CX and F-DX reduced 2.26 and 2.88 Log CFU/50 in^2 more of aerobic bacteria. respectively at 8 min in compare to their 5 min's treatments.

Table II

Survival of nature-borne bacteria (*Aerobic*, *Escherichia coli* and *Salmonella*) on bovine hide panels following the debris removing treatment.

Time of Treatment	Formulation	Bacterial populations recovered from haired surface of hide panels after treating with formulations (Log CFU/50 in^2)*		
		Aerobic bacteria colony count	*Escherichia coli* colony count	*Salmonella* colony count
8 min	F-A (control)	9.27±0.01^a	4.06±0.52^a	3.34±0.08^a
	F-B	7.70±0.21^b	3.70±0.12^a	0.15±0.27^{d,e}
	F-C	6.32±0.37^c	2.68±0.29^c	1.74±0.39^c
	F-D	3.84±0.06^a	2.90±0.09^c	1.25±0.24^c
	F-X	8.58±0.05^a	2.01±1.73^c	2.10±1.82^b
	F-BX	6.16±0.22^c	3.93±0.11^b	1.88±0.03^c
	F-CX	4.00±0.06^d	1.75±0.04^d	0.33±0.33^{d,e}
	F-DX	0.56±0.05^{e,f}	0.43±0.43^{e,f}	1.05±0.11^c
5 min	F-A (control)	9.11±0.01^a	4.19±0.52^a	3.46±0.08^a
	F-CX	6.26±0.33^{b,c}	3.71±0.04^b	2.11±0.06^{b,c}
	F-DX	3.44±0.42^d	2.63±0.13^c	0.33±0.33^d

*Results presented are a representation of triplicate calculation of bacterial population per sample.
The Dunnett's test was to evaluate the significance with confidence level was set to 95%; different letters within the same column indicate significant differences ($p < 0.05$).

Escherichia Coli Colony Count

In comparison with the control (F-A), inventive formulation resulted in maximum reduction of *Escherichia Coli* (*E. coli*) from the hide panels by 3.63 Log CFU/in^2 and minimum reduction of *E. Coli* by 0.13 Log CFU/in^2. The lowest *E. coli* population was counted from the hide panel treated with F-DX formulation at 8 min as expected. The addition of SDIC in formulation helped in further reduction of *E. coli* by 0.93 and 2.47 Log CFU/in^2 for F-CX and F-DX, in compare to F-C and F-D, respectively. However, hide panels were cut from a freshly flayed bovine hide collected from a local slaughter house and used for the experiments without any pretreatment to capture the real problem to be identified, therefore each piece of hide was loaded with different initial concentration of microorganism. Possibly for this reason, F-BX resulted in slightly higher colony count of *E. coli* than F-B. Treatment at 5 min with F-CX and F-DX also reduced *E.coli* population by 0.48 and 1.56 Log CFU/in^2, respectively in comparison to the 5 min-control. The bacterial population was reduced further by 1.96 and 2.2 Log CFU/in^2 when the hide panels were treated for 8 min instead of 5 min with F-CX and F-DX, respectively.

Salmonella Colony Count

As shown in Table II, similar results were obtained in recovery of *Salmonella* from the treated hide panels. All the formulations with/without SDIC were able to reduce *Salmonella* populations significantly. At 8 min treatment, F-B, F-C and F-D resulted in reduction of 3.19, 1.6 and 2.09 Log CFU/in^2, respectively in compare to the control (F-A). Treatment only with SDIC (F-X) was not effective as found in other cases. It only reduced 1.24 Log CFU/in^2 when compared to the water treatment (F-A). Formulations containing SDIC, F-BX, F-CX and F-DX offered reduction of *Salmonella* populations by 1.46, 3.01 and 2.29 Log CFU/in^2 respectively when compared to the control. Treatment with F-CX at 8 min reduced more *Salmonella* than its 5 min treatment as expected, however F-DX in 5 min showed better result over the 8 min treatment, was probably accounted for the difference in initial load of *Salmonella* on naturally collected hide surface.

Post-Leather Analysis

Bovine hide is a valuable byproduct as it produces leather which is a popular commodity. Therefore, it is important to evaluate that, any treatment on raw hides does not create any detrimental impact when the treated hide is converted into leather. From our previous experiment It was proven that the usage of SDIC alone on hide surface had no detrimental effect on leather quality (Sarker *et al*. 2020). For this study, all the leather panels produced from either formulation treated or control (water treated) hide samples were subjected under microscopic, organoleptic and mechanical property analysis for a side-by-side comparison. Microscopic analysis carryout out with a stereo microscope revealed (data is not included) no distinguishable difference on grain structures among

the leather pieces. Additionally, the leather panels were folded, and a stereo microscopic image was taken (data is not included) at the crease to observe if there was any sueding (fraying) from any of the samples. Again, there was no discernable difference between the experimental samples and the control. In Organoleptic analysis, crust leathers from formulation treated hides were assessed for softness, fullness, grain tightness (break), color and general appearance by hand and visual examination. This evaluation done by an USDA tanner exhibited similar subjective properties of all kind in comparison with the control. All this analysis suggests that the inventive formulations have no detrimental impact on subjective properties of finished leather.

Evaluation of leather panels for mechanical properties (Table III) revealed that, there was little to no difference on leather quality. Mechanical properties including tensile strength, elongation, Young's Modulus, and fracture energy of the leather samples produced from formulation treated hide samples (F-B, F-C, F-D, F-X, F-BX, F-CX and F-DX) were comparable to that produced from water washed hide panel (F-A, 8 min treated control was only evaluated). The little deviations in numbers can be attributed to naturally occurred uneven thickness of the bovine hide.

Conclusion

The removal of external debris from live cattle surface or at pre-eviscerated state of animal is a huge challenge for the meat industry. To clean the mud/manure debris is an essential task before meat processing as it relates to meat safety and also byproduct quality. The inventive formulation has been proven for its efficacy to remove external debris in such a short time that it has the potential to be used in industrial scale. The removal of attached debris from the haired surface facilitates the cleaning process of the animal carcass prior to removal of hide as it is shown in this study. Microbial populations, including nature-borne pathogens, were significantly reduced from the hide surface with the removal of debris when compared with water and also only antimicrobial solution treated hides. Therefore, the chance of cross-contamination of pathogens from hide to underlying meat will be minimized during meat processing. Also, the formulation treatment protects the hide's quality from being reduced during processing through the removal of adobe type of mud/manure balls which are firmly attached to the hair. The usage of formulation for such a short time on carcass surface has been demonstrated as non-detrimental for the valuable byproduct. This developed technology can potentially replace the current tedious and inefficient shaving or other conventional methods of removing attached debris from the cattle carcass which will save labor cost, utility cost and the most importantly reduce cross contamination during meat processing.

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Table III

Mechanical properties of crushed leather panels produced from water (control) and formulation treated hide pieces

Time of Treatment	Formulation	Tensile Strength (MPa)	Elongation, %	Young's Modulus (MPa)	Fracture Energy J/cm^3
8 min	F-A (control)	13.61 ± 2.32	45.26 ± 6.09	19.88 ± 5.15	1.58 ± 0.48
	F-B	15.154 ± 2.28	44.52 ± 7.25	14.99 ± 6.24	2.19 ± 0.70
	F-C	12.82 ± 2.00	45.03 ± 6.68	19.86 ± 2.7	2.37 ± 0.92
	F-D	15.35 ± 4.57	49.11 ± 6.25	18.50 ± 4.77	3.59 ± 1.49
	F-X	14.37 ± 2.13	46.23 ± 6.62	14.35 ± 7.19	2.08 ± 0.35
	F-BX	13.03 ± 1.28	46.48 ± 2.47	14.79 ± 0.98	1.40 ± 0.21
	F-CX	15.25 ± 6.15	38.72 ± 5.54	13.45 ± 2.24	1.86 ± 0.84
	F-DX	16.87 ± 1.31	42.43 ± 3.07	17.50 ± 8.52	2.32 ± 0.28
5 min	F-CX	14.65 ± 2.31	40.54 ± 5.75	13.62 ± 5.6	2.84 ± 0.53
	F-DX	15.39 ± 6.81	42.11 ± 7.65	11.87 ± 6.50	2.07 ± 1.00

Conflict of Interest

The authors declare that there is no conflict of interests on the work published in this paper.

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Efficient Removal of Ammonia-Nitrogen from Deliming Effluent by using Magnesium Ammonium Phosphate Precipitation Method

by

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Abstract

Ammonia nitrogen (NH_3-N) pollutant has received much attention in the leather industry. An efficient strategy for removal of NH_3-N from tannery wastewater was proposed by using a magnesium ammonium phosphate (MAP) precipitation method to remove NH_3-N from deliming effluent with the highest NH_3-N concentration among all leather-making effluents. Results showed that approximately 80% of NH_3-N was removed from deliming effluent when reacting at P/Mg/N mole ratio of 1.2:1.2:1.0 and pH 9.5 for 20 min. The NH_3-N and total nitrogen concentrations of tannery wastewater (a composite wastewater of all leather-making effluents) sharply decreased by treating deliming effluent with MAP precipitation, which greatly improved the biological treatment efficiency of tannery wastewater. The residual concentration of NH_3-N in the treated tannery wastewater was less than 2 mg/L. The total phosphorus concentration of tannery wastewater increased by less than 0.4 mg/L, indicating that secondary pollution of phosphorus did not occur after MAP precipitation treatment.

Introduction

Beamhouse procedures are important for the removal of preserving salts, dirt, blood, hair, epidermis, non-structural proteins, and fat from raw hide and dispersion of hide collagen fibers.[1] However, the beamhouse procedures cause pollution in tannery wastewater. For example, deliming and bating procedures generally use ammonium salts such as $(NH_4)_2SO_4$ and NH_4Cl, resulting in high concentration of ammonia nitrogen (NH_3-N) in deliming and bating effluents (2,000–4,000 mg/L). The NH_3-N amount of deliming and bating effluents accounts for 60%–70% of the NH_3-N amount of tannery wastewater that includes all effluents released from leather-making procedures.[2] The high concentration of NH_3-N in deliming and bating effluents contributes to a low C/N ratio of the tannery wastewater, making it difficult to achieve an efficient biological treatment of tannery wastewater and a satisfactory NH_3-N removal efficiency.[3] The high NH_3-N load in deliming and bating effluents deteriorates the tannery wastewater. If the tannery wastewater contains a large amount of NH_3-N, it will cause the eutrophication of water, the low concentration of dissolved oxygen in water, and the death of fish and other organisms.[4,5]

NH_3-N pollution caused by deliming and bating can be eliminated by replacing ammonium salts with ammonium-free deliming agents. However, existing ammonium-free deliming agents, such as carbon dioxide,[6,7] acetic acid, lactic acid, citric acid,[8,9] magnesium salts,[10] peracetic acid,[11] glycolic acid,[12] and glycine,[13] have small pH-buffering capacity, slow penetration in limed pelt, or high cost. Consequently, ammonium-free deliming agents only have small-scale applications, and ammonium salts with excellent deliming performance and low price are still popular for deliming and bating procedures in tanneries. Thus, an effective way to reduce the NH_3-N in tannery wastewater is the removal of NH_3-N from the deliming and bating effluents with high NH_3-N concentration and small volume before the two effluents are released into tannery wastewater.

Many methods involving ammonia stripping,[14,15] ion exchange method,[16,17] membrane technology,[18,19] and chlorination[20,21] have been developed to remove NH_3-N from wastewater. However, the ammonia stripping method requires large-scale heating to strip ammonia and strong acid solution (mainly H_2SO_4) to absorb ammonia.[22] The ion exchange method is generally used to treat the wastewater with low concentration of NH_3-N because the ion exchange material usually shows a limited exchange capacity.[23] For the membrane technology, membranes are easily damaged by suspended solids in deliming and bating effluents. Chlorination uses a lot of chlorine and produces harmful byproducts.[21] Therefore, these methods are not appropriate for the treatment of deliming and bating effluents.

The magnesium ammonium phosphate (MAP) precipitation method removes high concentration of NH_3-N from various wastewaters, such as aquaculture wastewater,[24] landfill leachate,[25] and human urine.[26] The formation of MAP proceeds through the following reactions:[27]

$$Mg^{2+} + NH_4^+ + HPO_4^{2-} + 6H_2O \rightarrow MgNH_4PO_4 \cdot 6H_2O + H^+$$

$$Mg^{2+} + NH_4^+ + H_2PO_4^- + 6H_2O \rightarrow MgNH_4PO_4 \cdot 6H_2O + 2H^+$$

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MAP precipitation method requires the addition of magnesium salt and phosphate into wastewater to make NH_3-N precipitate. The addition of phosphate has received considerable attention because it usually results in excessive phosphorus residue in wastewater after MAP precipitation treatment. The secondary pollution of phosphorus limits the application of MAP precipitation method in wastewater treatment. In fact, Tünay *et al.* reported NH_3-N removal with MAP precipitation in a composite wastewater of liming, deliming and bating effluents and tannery wastewater (a composite wastewater of all leather-making effluents).[28] However, the liming effluent with high sulfide and calcium concentrations made the composite wastewater undergo complex pretreatment and difficult to simultaneously achieve a high NH_3-N removal efficiency and a low residual phosphorus concentration. Additionally, the MAP precipitation treatment of the tannery wastewater was not economical and led to a high residual concentration of phosphorus in the treated wastewater (ranging from 19.3 mg/L to 199 mg/L) because the tannery wastewater has large volume and relatively low concentration of NH_3-N. It is well known that the NH_3-N pollution mainly comes from deliming and bating procedures rather than the other leather-making procedures. The deliming and bating effluents have lower sulfide and calcium concentrations than the liming effluent and much smaller volume than the tannery wastewater. Moreover, the use of phosphorus-containing chemicals in the whole leather-making processes is limited. Therefore, the introduction of phosphorus into deliming and bating effluents caused by MAP precipitation treatment will scarcely affect the total phosphorus (TP) concentration and C/N/P ratio of tannery wastewater. It is reasonable to speculate that MAP precipitation method can effectively remove NH_3-N from deliming and bating effluents without the secondary pollution of phosphorus. Besides, the MAP precipitate formed by treating the NH_3-N in the deliming and bating effluents can even be reused as slow-release fertilizer.[29]

This study aims to effectively remove NH_3-N from deliming and bating effluents by using the MAP precipitation method. The effects of molar ratio of HPO_4^{2-} to NH_4^+, molar ratio of Mg^{2+} to NH_4^+, reaction pH, and reaction time on NH_3-N removal efficiency and residual phosphorus concentration of deliming and bating effluents were investigated. Finally, the effect of the treatment of deliming and bating effluents with MAP precipitation on the biological treatment of tannery wastewater was investigated.

Experimental

Materials

Deliming effluent with pH of 8.72 and NH_3-N concentration of 2,105 mg/L was collected after deliming. Bating effluent with NH_3-N concentration of 776 mg/L was also collected after bating. Analytical-grade $MgCl_2{\cdot}6H_2O$ and $Na_2HPO_4{\cdot}12H_2O$ were obtained from Kelong Chemical Reagent Factory (Chengdu, China). High-range NH_3-N and TP test reagents were obtained from Fuzhou Fuguang Water Science & Technology Co., Ltd. (Fuzhou, China). Activated sludge used for biological treatment of tannery wastewater was provided by Chongzhou Fubang Leather Co., Ltd. (Chengdu, China).

Effect of pH adjustment method on NH_3-N removal efficiency

At room temperature, 2.43 g $Na_2HPO_4{\cdot}12H_2O$ and 1.51 g $MgCl_2{\cdot}6H_2O$ (P-to-Mg-to-N mole ratio [P/Mg/N] of 1.1:1.2:1.0) were added to 50 mL of deliming effluent. The pH of deliming effluent was separately adjusted using three different methods as follows: (1) no adjustment (control); (2) pH adjusted to 9.0 by using 1 mol/L NaOH solution before adding $Na_2HPO_4{\cdot}12H_2O$ and $MgCl_2{\cdot}6H_2O$ (Method A); and (3) pH maintained at 9.0 by using 1 mol/L NaOH solution after adding $Na_2HPO_4{\cdot}12H_2O$ and $MgCl_2{\cdot}6H_2O$ (method B). The reaction was performed with magnetic stirring for 15 min and standing for 30 min. Then, the concentration of NH_3-N in the supernatant of the reaction mixture was determined with high-range NH_3-N test reagent and water quality analyzer (DR6000, Hach, USA) according to the salicylic acid method (Method 10031).[30] The NH_3-N removal efficiency was calculated using Equation (1) as follows:

$$\text{NH}_3\text{-N removal efficiency} = \frac{X\text{-}Y}{X} \times 100\% \quad (1)$$

where X is the concentration of NH_3-N in deliming effluent before treatment, and Y is the concentration of NH_3-N in the supernatant of deliming effluent after treatment.

Optimization of MAP precipitation method for removal of NH_3-N from deliming effluent

Batch experiments were conducted at room temperature to investigate the effects of molar ratio of P to N (P/N), molar ratio of Mg to N (Mg/N), reaction pH, and reaction time on NH_3-N removal efficiency and TP concentration of deliming effluent after MAP precipitation treatment.

Effect of P/N

Different dosages of $Na_2HPO_4{\cdot}12H_2O$ (P/N 0.9, 1.0, 1.1, 1.2, 1.3 and 1.4) and 1.51 g $MgCl_2{\cdot}6H_2O$ (Mg/N, 1.2) were added into 50 mL of deliming effluent, and the reaction was performed at pH 9 with magnetic stirring for 15 min. After magnetic stirring, the reaction mixtures were left standing for 30 min, and the supernatants were collected to determine the NH_3-N and TP concentrations. To determine the TP concentration of the supernatant, we first digested the supernatant with potassium persulfate at 150°C for 30 min by using a digester (DRB200, Hach, USA). Then, the TP concentration of supernatant was measured using the TP test reagent and water quality analyzer according to the digestion-molybdenum antimony method (Method 10127).[30]

Effect of Mg/N

Different dosages of $MgCl_2 \cdot 6H_2O$ (Mg/N, 0.9, 1.0, 1.1, 1.2, 1.3 and 1.4) and 2.66 g $Na_2HPO_4 \cdot 12H_2O$ (P/N, 1.2) were added into 50 mL of deliming effluent, and the reaction was performed at pH 9 with magnetic stirring for 15 min. After magnetic stirring, the reaction mixtures were left standing for 30 min. Then, the NH_3-N and TP concentrations of supernatants were determined.

Effect of reaction pH

After adding 2.66 g $Na_2HPO_4 \cdot 12H_2O$ and 1.51 g $MgCl_2 \cdot 6H_2O$ (P/Mg/N, 1.2:1.2:1.0) into 50 mL of deliming effluent, the pH of effluent was adjusted to a preset value (8.0, 8.5, 9.0, 9.5, 10.0, and 10.5) and then maintained throughout the reaction (magnetic stirring for 15 min). After the reaction, the mixtures were left standing for 30 min to determine the NH_3-N and TP concentrations of supernatants.

Effect of reaction time

The MAP precipitation reaction of deliming effluent was performed at pH 9.5 and P/Mg/N of 1.2:1.2:1.0 with magnetic stirring for 10, 20, 30, 40, 50, and 60 min. Then, the reaction mixtures were left standing for 30 min to determine the NH_3-N and TP concentrations of supernatants.

X-ray diffraction analysis of precipitate

The precipitate from deliming effluent that was coprecipitated with $Na_2HPO_4 \cdot 12H_2O$ and $MgCl_2 \cdot 6H_2O$ was collected after reacting with P/Mg/N at a ratio of 1.2:1.2:1.0 and pH 9.5 for 20 min. The precipitate was washed thrice with distilled water and then dried at 45°C for 48 h to analyze the specific composition using an X-ray diffractometer with *Cu* Kα radiation ($\lambda = 1.5406$ Å; DX-2700, Haoyuan, China).

Aerobic biological treatment of simulated tannery wastewater

Three kinds of simulated tannery wastewaters were prepared for aerobic biological treatment experiment. A simulated tannery wastewater (control group) was prepared by mixing the effluents from each operation, except for chrome tanning, based on the amount of water used in each operation (Table I). Two other simulated tannery wastewater samples (experiment groups) were prepared using the same procedures, except that the deliming or bating effluents were treated with MAP precipitation before mixing

Table I

Wastewater sampling from leather-making procedures

No.	Procedure	Sampling volume (L)		
		Control	Experiment 1	Experiment 2
1	Presoaking	2	2	2
2	Washing	2	2	2
3	Soaking	2	2	2
4	Liming	3.8	3.8	3.8
5	Washing	3	3	3
6	Deliming	1	1 (MAP treatment)	1 (MAP treatment)
7	Bating	1	1	1 (MAP treatment)
8	Washing (3 times)	9	9	9
9	Pickling	0.5	0.5	0.5
10	Chrome tanning	No sampling	No sampling	No sampling
11	Washing	3	3	3
12	Rewetting	3	3	3
13	Washing	3	3	3
14	Neutralization	2	2	2
15	Washing	3	3	3
16	Retanning & Dyeing	1	1	1
17	Fatliquoring & Adding	1	1	1
18	Washing (3 times)	9	9	9
Total		49.3	49.3	49.3

into tannery wastewater (Table I). For experiment 1, the deliming effluent was treated at P/Mg/N mole ratio of 1.2:1.2:1.0, pH 9.5, and room temperature for 20 min. For experiment 2, the deliming and bating effluents were treated at P/Mg/N mole ratio of 1.2:1.2:1.0, pH 9.5, and room temperature for 20 min.

The three simulated tannery wastewaters were separately adjusted to pH 7.5 by using 2 mol/L sulfuric acid solution, mixed with activated sludge at a volume ratio of 3:1 (wastewater: sludge), and placed in a 2 L glass beaker. An aeration device (ACO-003, SunSun, China) was used to aerate the mixtures of activated sludge and simulated tannery wastewater at 25°C for aerobic biological treatment. After treatment for 0, 4, 8, 12, and 24 h, the concentration of dissolved oxygen (DO) was measured using a DO meter (HI9146, Hana, Italy), and the concentrations of mixed liquor suspended solids (MLSS) and sludge volume (SV) in the mixture of wastewater and sludge were determined using standard methods.[31] Moreover, the mixtures were centrifuged at 5,000 rpm for 10 min, and the supernatants were used to determine the NH_3-N, total organic carbon (TOC), total nitrogen (TN), and TP concentrations. TOC and TN concentrations were determined using TOC/TN analyzer (Vario TOC, Elementar, Germany).

Results and Discussion

Effect of pH adjustment method on NH_3-N removal efficiency

According to the reaction equation of MAP formation (see Introduction section), the MAP precipitation process will release H^+, causing a decrease in the pH of reaction system. The consumption of H^+ helps the precipitation reaction proceed in the forward direction, which can increase the removal efficiency of NH_3-N. Therefore, we first investigated the effect of pH regulation on the removal of NH_3-N from deliming effluent by using MAP precipitation method. The data in Table II show that the pH of deliming effluent sharply decreased to approximately 5.5 after the MAP precipitation reaction when the initial pH of deliming effluent was not adjusted (control) or just adjusted to 9.0 by using a small amount of NaOH before adding $Na_2HPO_4{\cdot}12H_2O$ and $MgCl_2{\cdot}6H_2O$ (Method A). The removal efficiencies of NH_3-N from deliming effluent by using pH adjustment Methods control and A were as low as approximately 50%. This was because the high concentration of NH_3-N in the deliming effluent made the MAP formation release a large amount of H^+ at the initial stage of precipitation reaction. Subsequently, the pH of the reaction system changed from alkaline to acidic pH, and this condition inhibited the continuous formation of MAP.[28] For Method B, the pH of the reaction system was maintained at 9.0 by continuously adding NaOH solution during the precipitation reaction. Thus, MAP precipitation was carried out more thoroughly, and the removal efficiency of NH_3-N was greatly increased to 78%. These results indicated that the pH of the reaction system mainly affects the removal of NH_3-N from wastewater by MAP precipitation, supporting the results obtained in previous studies.[32,33] Keeping the pH of reaction system in an alkaline pH range is important for the efficient removal of NH_3-N from deliming effluent.

Effects of reaction conditions on the removal of NH_3-N from deliming effluent by using MAP precipitation method

MAP precipitation reaction conditions such as reaction pH, molar ratio of HPO_4^{2-} to Mg^{2+} to NH_4^+, and reaction time influence the removal efficiency of NH_3-N and the composition and crystal structure of products. This section presents the effects of P/N, Mg/N, reaction pH, and reaction time on the removal of NH_3-N from deliming effluent. The TP concentration of deliming effluent after MAP precipitation treatment was also measured to determine whether secondary pollution of phosphorus occurred.

Table II

Effect of pH adjustment method on NH_3-N removal efficiency

pH adjustment method	pH of deliming effluent after reaction	NH_3-N removal efficiency
Control	5.47±0.01	48.01±1.34%
Method A	5.48±0.01	50.20±1.54%
Method B	9.00	77.87±1.19%

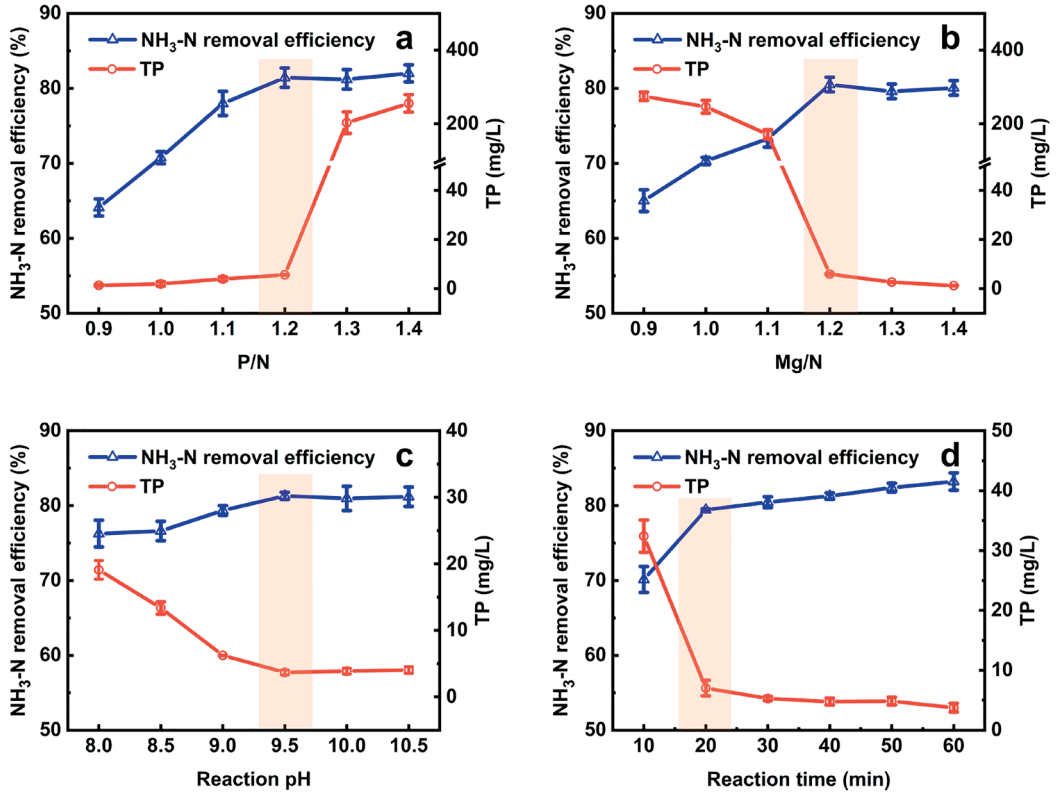

Figure 1. Effects of P/N **(a)**, Mg/N **(b)**, reaction pH **(c)**, and reaction time **(d)** on the removal of NH_3-N from deliming effluent and residual concentration of TP in treated deliming effluent by using MAP precipitation method

Figure 1(a) shows the effect of P/N on NH_3-N removal efficiency and residual TP concentration of treated deliming effluent under Mg/N ratio of 1.2, reaction pH of 9.0, and reaction time of 15 min. When the P/N increased from 0.9 to 1.2, the NH_3-N removal efficiency increased from 64.11% to 81.42%, and the residual TP concentration in the treated deliming effluent increased from 1.4 mg/L to 5.6 mg/L. When the P/N continued to increase to 1.3, the NH_3-N removal efficiency scarcely increased, and the TP concentration rapidly increased and exceeded 200 mg/L. This phenomenon occurred because when the P/N was higher than 1.2, free NH_3-N was mostly consumed, and the excess phosphate could not coprecipitate with NH_3-N and remained in the deliming effluent. Therefore, the optimal P/N was 1.2, considering the chemical cost and phosphorus pollution.

The data in Figure 1(b) show that when the Mg/N increased from 0.9 to 1.2 (P/N=1.2, pH 9.0, reaction time 15 min), the NH_3-N removal efficiency increased from 65.02% to 80.50%, and the residual TP concentration decreased from 274.9 mg/L to 6.0 mg/L. However, almost no increase was observed in the NH_3-N removal efficiency, and the TP concentration slightly decreased when the Mg/N continued to increase. Thus, the optimal Mg/N of 1.2 was selected.

An alkaline pH of reaction system was conducive to the precipitation of MAP.[34] Therefore, the effect of reaction pH in the range of 8.0–10.5 on NH_3-N removal efficiency and TP concentration of treated deliming effluent were investigated. As shown in Figure 1(c), at P/Mg/N ratio of 1.2:1.2:1.0 and reaction time of 15 min, the NH_3-N removal efficiency increased from 76.26% to 81.31% with the increase in pH from 8.0 to 9.5, and the TP concentration decreased from 19.1 mg/L to 3.7 mg/L. However, higher NH_3-N removal efficiency and lower TP concentration were not obtained by continuously increasing the reaction pH. Hence, the reaction pH of 9.5 was appropriate.

The effect of reaction time on NH_3-N removal and residual phosphorus at P/Mg/N ratio of 1.2:1.2:1.0 and reaction pH of 9.5 is shown in Figure 1(d). The reaction almost reached an equilibrium within 20 min at room temperature. The NH_3-N removal efficiency was as high as 79.44%, and the residual TP concentration was as low as 7.0 mg/L after reacting for 20 min. The results indicated that MAP precipitate rapidly formed at the initial stage of reaction, which was consistent with kinetics of crystallization and dissolution of MAP.[35,36]

Taken together, the MAP precipitation method was feasible for the removal of high-concentration NH_3-N from deliming effluent within a short time at room temperature. The optimized precipitation conditions were P/N of 1.2, Mg/N of 1.2, reaction pH of 9.5, and reaction time of 20 min. Moreover, the residual phosphorus in the treated deliming effluent introduced by MAP precipitation method slightly influenced the TP concentration of tannery wastewater because of the small volume ratio of deliming effluent to tannery wastewater.

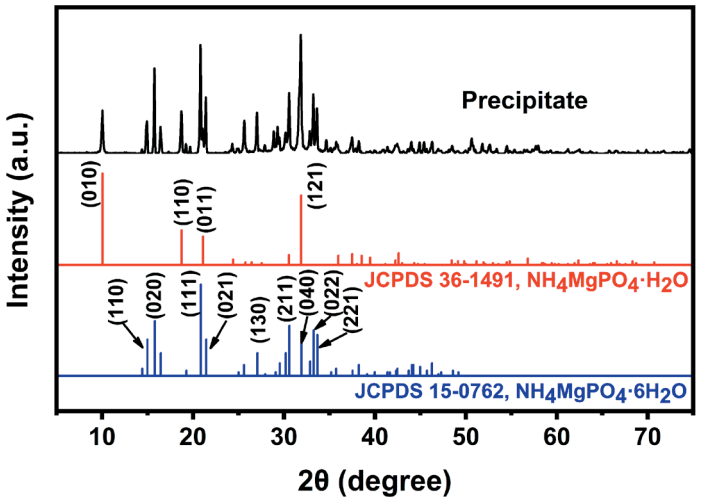

Figure 2. XRD pattern of the precipitate obtained from MAP precipitation treatment of deliming effluent

Characterization of precipitation

The precipitate formed by the MAP precipitation treatment of deliming effluent was collected under the optimum reaction conditions (P/N=1.2, Mg/N=1.2, pH 9.5, reaction time 20 min). The precipitate was first washed to remove the free organic matter and soluble salts and then analyzed by X-ray diffraction. As shown in Figure 2, the XRD pattern of precipitate shows sharp peaks, indicating that the precipitate was well crystallized.[37] Based on the comparison of the XRD pattern of precipitate and the standard XRD patterns of synthetic struvite (JCPDS Card No. 36-1491) and synthetic dittmarite (JCPDS Card No. 15-0762), the precipitate is a mixture of struvite and dittmarite. This result proved that the NH_3-N in the deliming effluent coprecipitated with $MgCl_2·6H_2O$ and $Na_2HPO_4·12H_2O$ and formed crystalline MAP monohydrate and hexahydrate.

Effect of MAP precipitation method on the biological treatment of tannery wastewater

This work aims to propose an effective method to achieve satisfactory biological treatment and NH_3-N removal of tannery wastewater. In this section, we investigated how the MAP precipitation treatment of deliming and bating effluents affects the aerobic biological treatment and pollution loads of tannery wastewater.

The concentrations of NH_3-N, TOC, TN, and TP in the control and experimental simulated tannery wastewaters are shown in Table III. The NH_3-N concentrations of Experiments 1 and 2 tannery wastewaters were merely 58.36% and 42.72% of the control one, respectively. This indicated that the treatment of high-concentration NH_3-N-containing deliming and bating effluents with MAP precipitation method greatly reduced the concentration of NH_3-N in the tannery wastewater. Considering that NH_3-N is an important source of nitrogen in tannery wastewater, the TN concentrations of experimental tannery wastewaters decreased with the decrease in NH_3-N concentration.[38] Although some phosphate was added into the deliming and bating effluents to remove NH_3-N, the TP concentrations of experimental tannery wastewaters slightly increased compared with the control one. This phenomenon occurred, because the TP concentrations of deliming and bating effluents after MAP precipitation treatment were lower than 7 mg/L. Moreover, the volume ratio of deliming and bating effluents to tannery wastewater was as small as 1:24. The sharp decrease in the initial TN concentration and the slight increase in the initial TP concentration of tannery wastewater caused by the MAP precipitation treatment of deliming and bating effluents improved the C: N: P ratio of tannery wastewater and should be beneficial to the biological treatment.

The simulated tannery wastewaters were subsequently treated with activated sludge to evaluate the performance of aerobic biological

Table III

Concentrations of NH_3-N, TOC, TN, and TP in simulated tannery wastewater before and after aerobic biological treatment

Simulated tannery wastewater		NH_3-N(mg/L)	TOC (mg/L)	TN (mg/L)	TP (mg/L)
Before treatment	Control	102.3±4.8	851.1±13.7	260.3±14.8	5.9±0.2
	Experiment 1	59.7±1.8	777.6±25.7	181.5±13.2	6.3±0.3
	Experiment 2	43.7±1.3	705.2±19.9	144.9±6.7	7.0±0.3
After treatment	Control	35.9±4.3	152.9±5.4	130.9±8.5	0.4±0.1
	Experiment 1	2.0±0.2	164.6±6.2	74.6±6.2	0.5±0.1
	Experiment 2	1.5±0.1	177.7±8.0	64.9±5.7	0.8±0.1

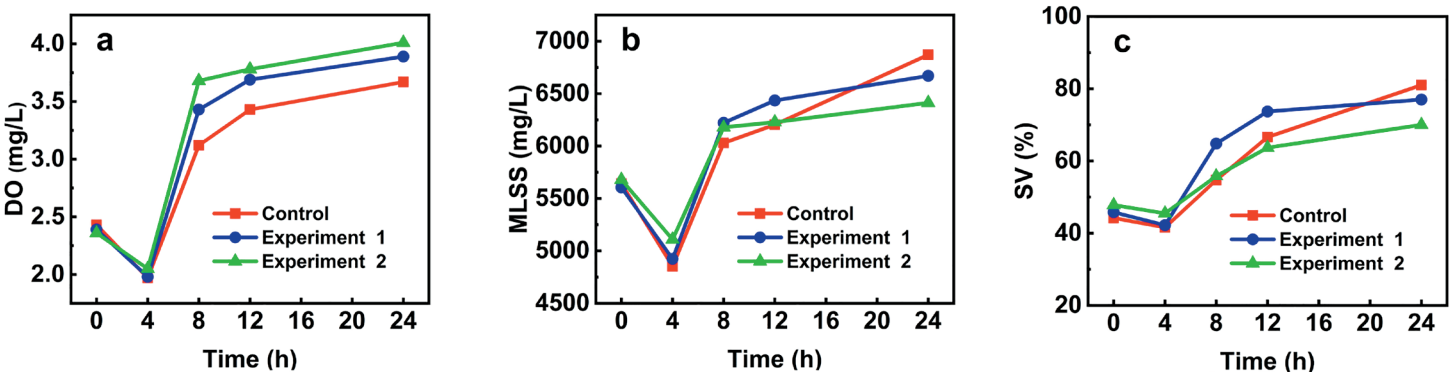

Figure 3. DO **(a)**, MLSS (**b**) and SV **(c)** of activated sludge during aerobic biological treatment

treatment. During the treatment process, the DO, MLSS and SV concentrations were within normal ranges (Figure 3), indicating that the aerobic microorganisms in activated sludge grew well after cultivation and domestication.[39-41] The concentrations of NH_3-N in the two experimental tannery wastewaters decreased to less than 25 mg/L within 8 h, and reached a very low level (below 2 mg/L) after treatment for 24 h, whereas the NH_3-N concentration of the control group was 35.9 mg/L after treatment for 24 h (Fig. 4a and Table III). The TOC concentrations of the three tannery wastewaters decreased to 150–180 mg/L after treatment for 24 h (Fig. 4b). The residual TN concentrations of experimental groups after aerobic biological treatment were close to half of the control one (Fig. 4c). The low TN concentrations of experimental tannery wastewaters after aerobic biological treatment are helpful for the subsequent anaerobic denitrification of tannery wastewater and complete and rapid TN removal.[42] Figure 4(d) shows that although the initial concentrations of TP in experimental wastewaters were higher than the control group, they decreased more rapidly during treatment process. Additionally, the TP concentrations of experimental wastewaters were lower than 1 mg/L after treatment for 12 h, whereas the control group required a longer time to reach this TP concentration.

Overall, the aerobic biological treatment effects of the two experimental tannery wastewaters were better than that of the control group, because the MAP precipitation treatment of deliming and bating effluents decreased the initial NH_3-N and TN concentrations of wastewater and improved the C:N ratio for biological treatment.

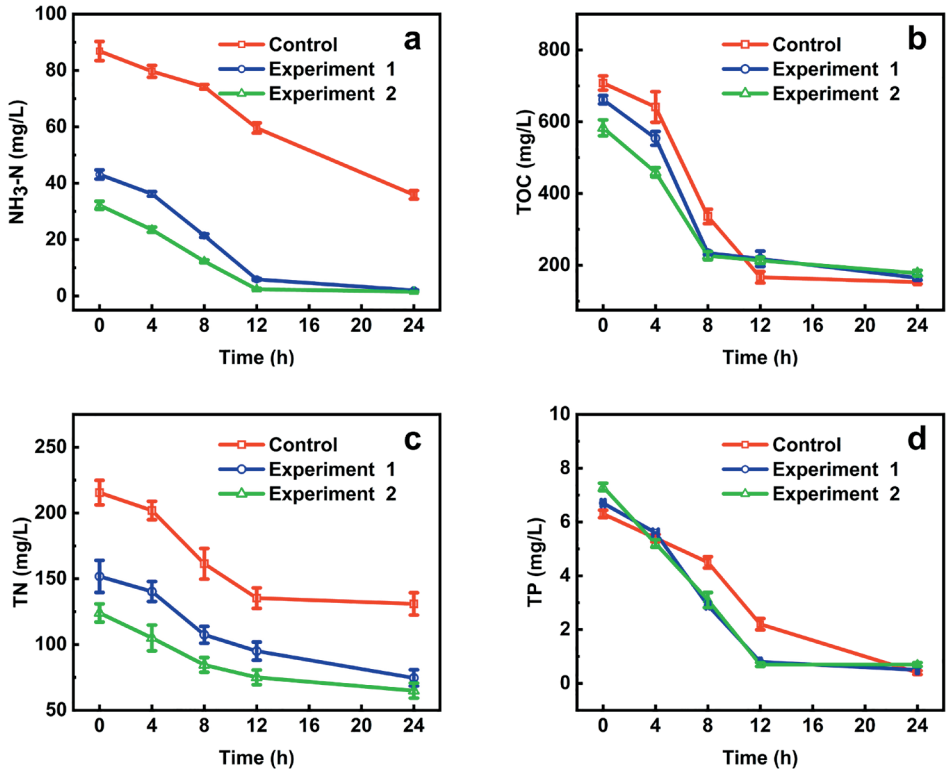

Figure 4. Effect of MAP precipitation method of deliming and bating effluents on aerobic biological treatment of simulated tannery wastewater: **(a)** NH_3-N concentration; (**b**) TOC concentration; **(c)** TN concentration; and **(d)** TP concentration. Upon the addition of activated sludge, the initial NH_3-N, TOC, TN, and TP concentrations of simulated tannery wastewaters were slightly lower than the values shown in Table 3.

Conclusions

The MAP precipitation method is suitable for the treatment of deliming and bating effluents with high concentration of NH_3-N and small volume. An efficient removal of NH_3-N from deliming effluent and a low concentration of residual phosphorus in the treated deliming effluent were obtained under the optimized experimental condition. The sharp decrease in the NH_3-N concentration of deliming and bating effluents dramatically reduced the NH_3-N and TN concentrations of tannery wastewater and remarkably improved the C:N:P ratio of tannery wastewater and the efficiency of biological treatment. Therefore, the MAP precipitation treatment of deliming and bating effluents can effectively remove NH_3-N from tannery wastewater without introducing secondary pollution of phosphorus. This work provides new insights into the efficient removal of NH_3-N and TN from tannery wastewater.

Acknowledgements

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

by

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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

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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.

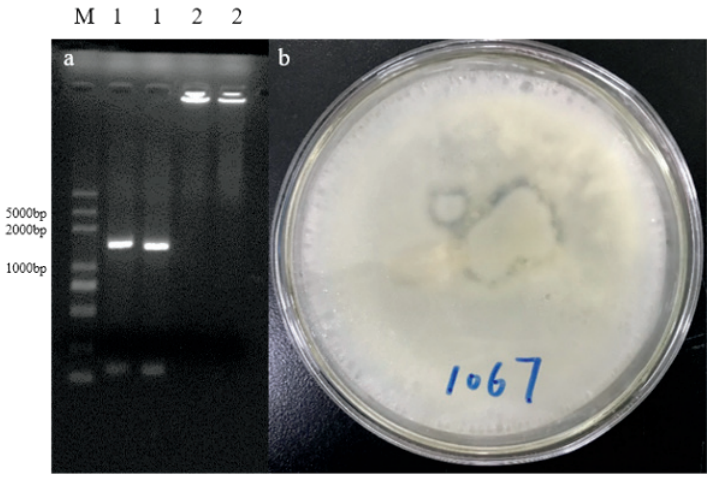

Figure 1. Construction of plasmid polymers and expression of protease. **a:** Construction of recombinant plasmid and verification of successful introduction of protease gene, M: marker, 1: metalloprotease 1067 gene fragment, 2: plasmid polymers; **b:** Hydrolytic circle of protease 1067

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

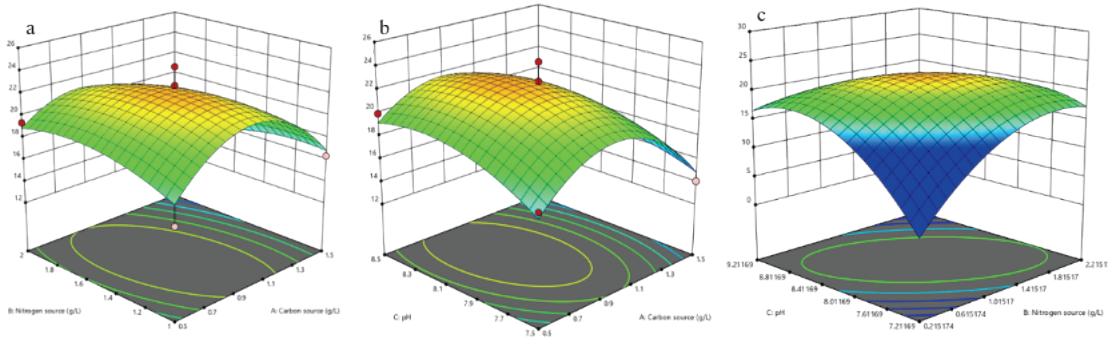

Figure 2. 3D surface of three-level and three-factor response surface.

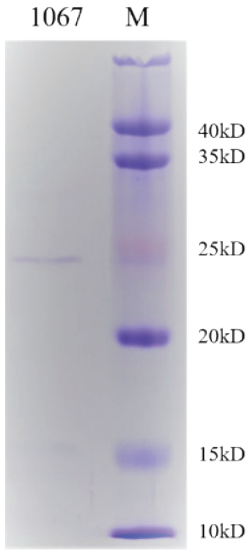

Figure 3. SDS-PAGE of purified protease 1067. M: Marker, 1067: protease 1067

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

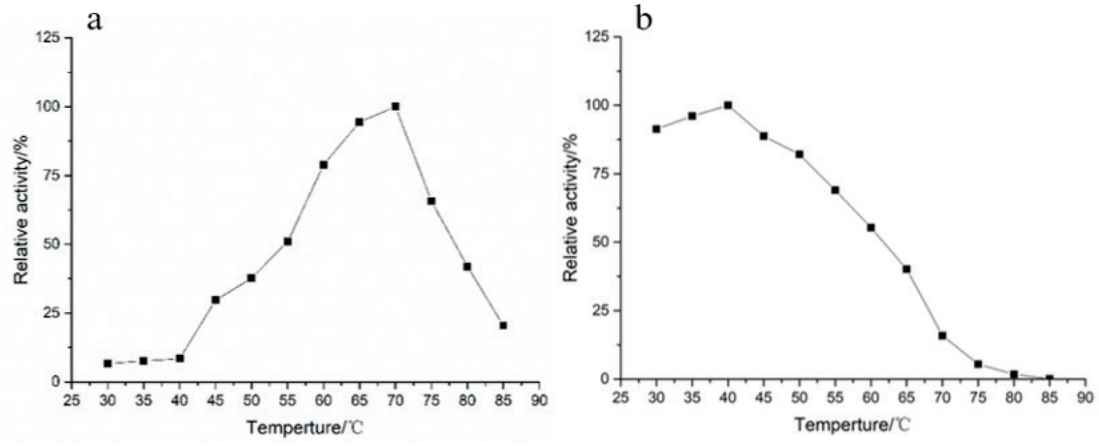

Figure 4. Effect of temperature on enzyme activity and stability. **a:** effect of temperature on enzyme activity; **b:** effect of temperature on enzyme stability

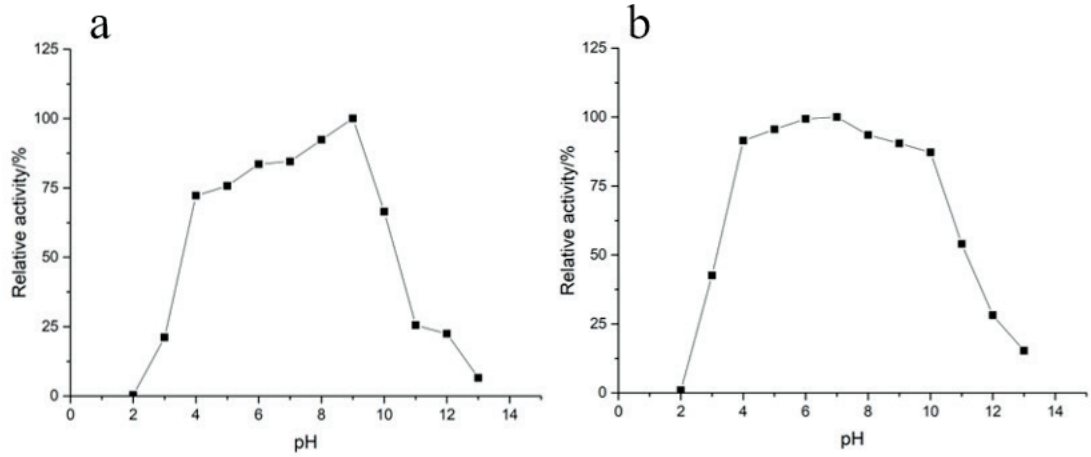

Figure 5. Effect of pH on enzyme activity and stability. **a:** effect of pH on enzyme activity; **b:** effect of pH on enzyme stability

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

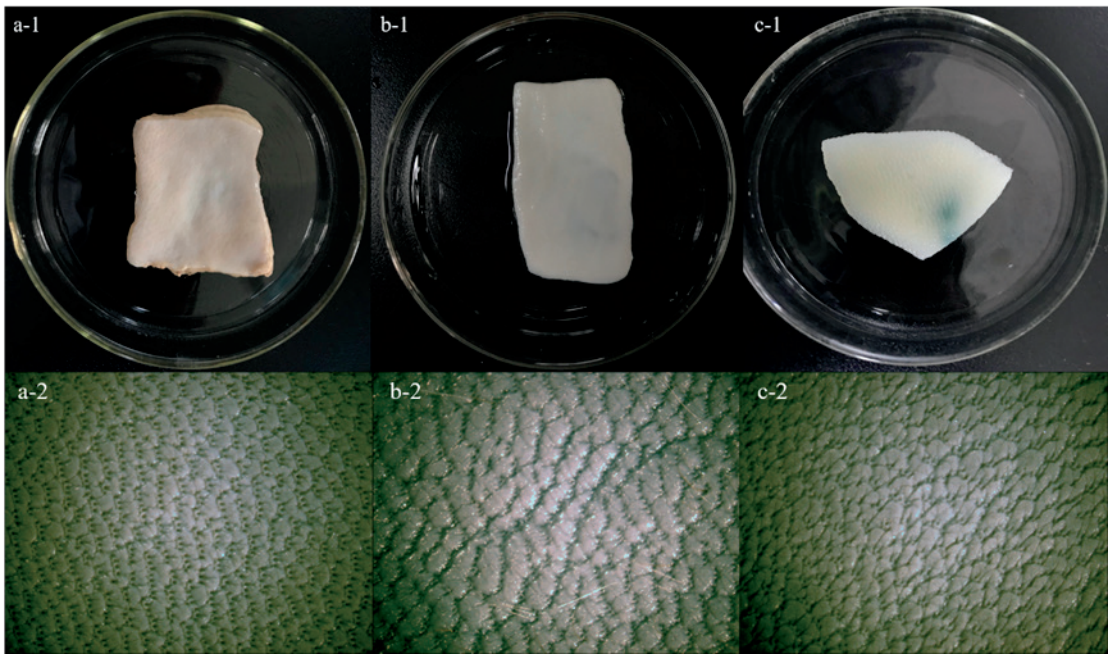

Figure 6. 1: Unhairing performance on goatskins by different unhairing methods.
2: The grain surface of pelts with different unhairing methods.
a: unhairing by protease 1067; **b:** unhairing by protease AS1.398; **c:** unhairing by Na_2S

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,

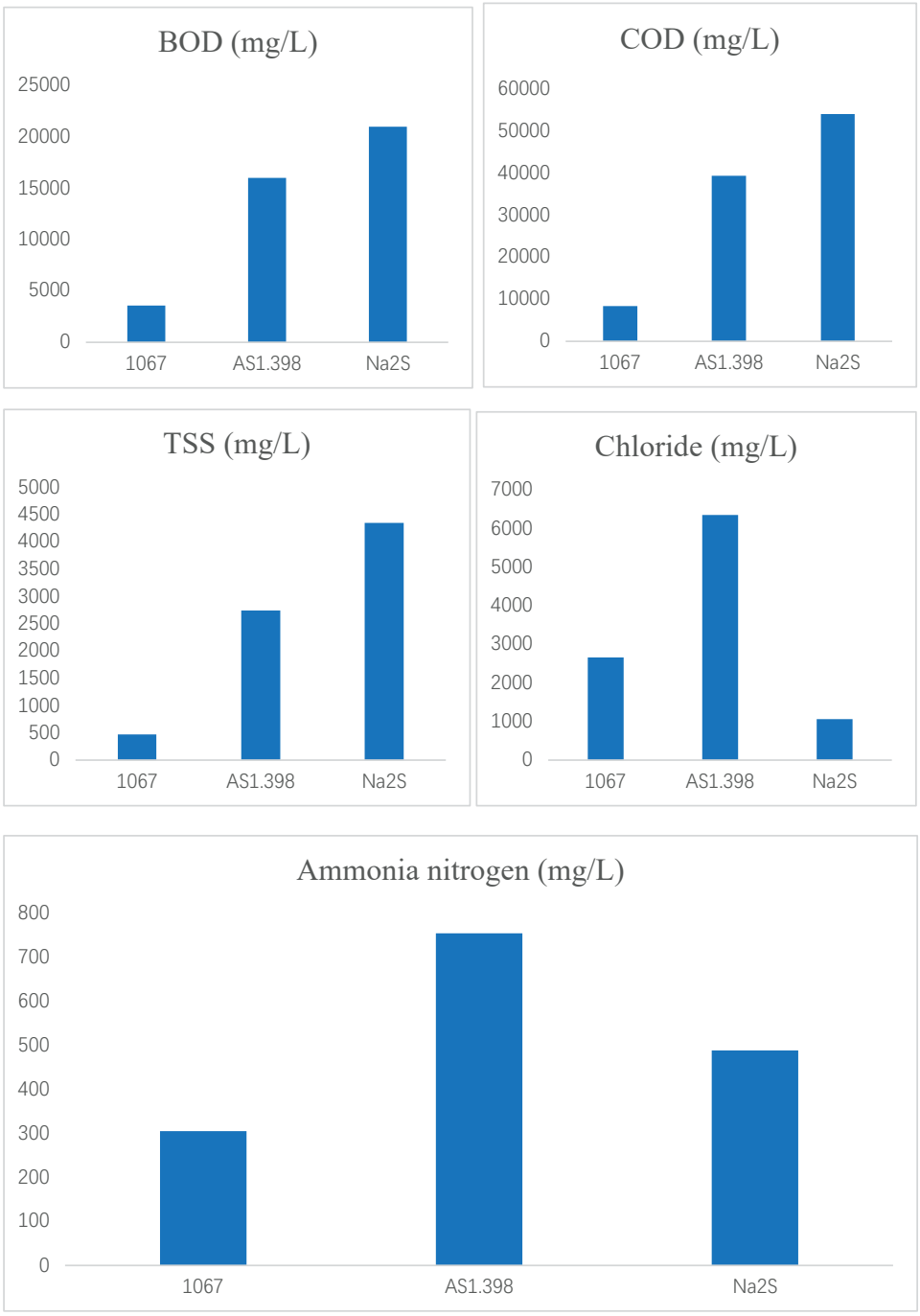

Figure 7. The content of main substances in the wastewater after unhairing

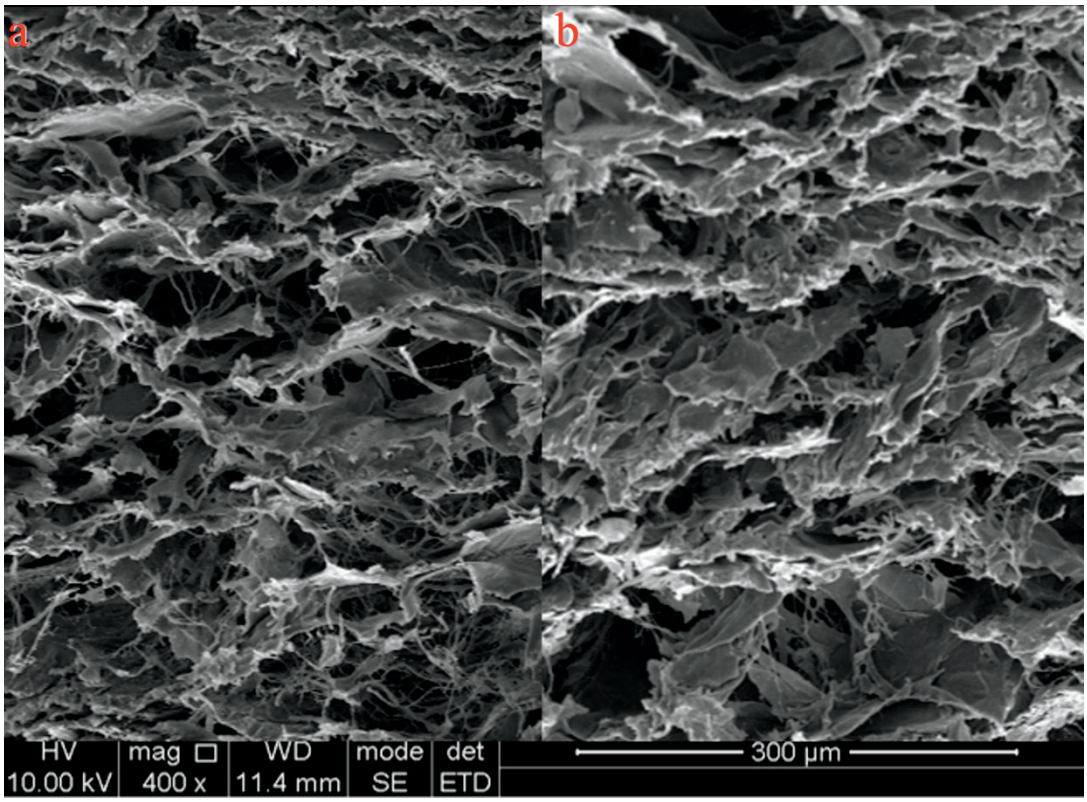

Figure 8. The cross section of dehaired skin detected by SEM.
a: enzymatic unhairing; **b:** conventional chemical unhairing

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]

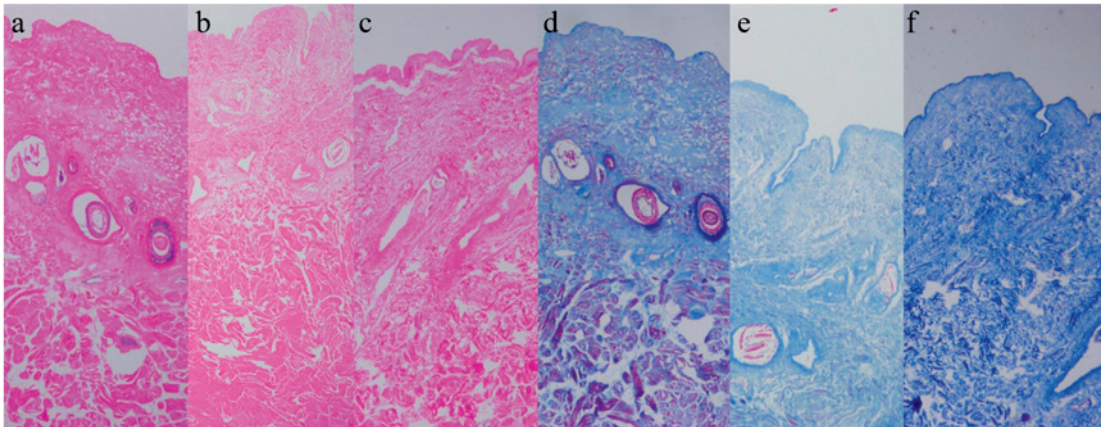

Figure 9. Staining results of bare skin. **a, b, c:** The results of H&E-staining of goat skin treated with Na_2S, AS1.398 and 1067; **d, e, f:** The results of Masson-staining of goat skin treated with Na_2S, AS1.398 and 1067

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.

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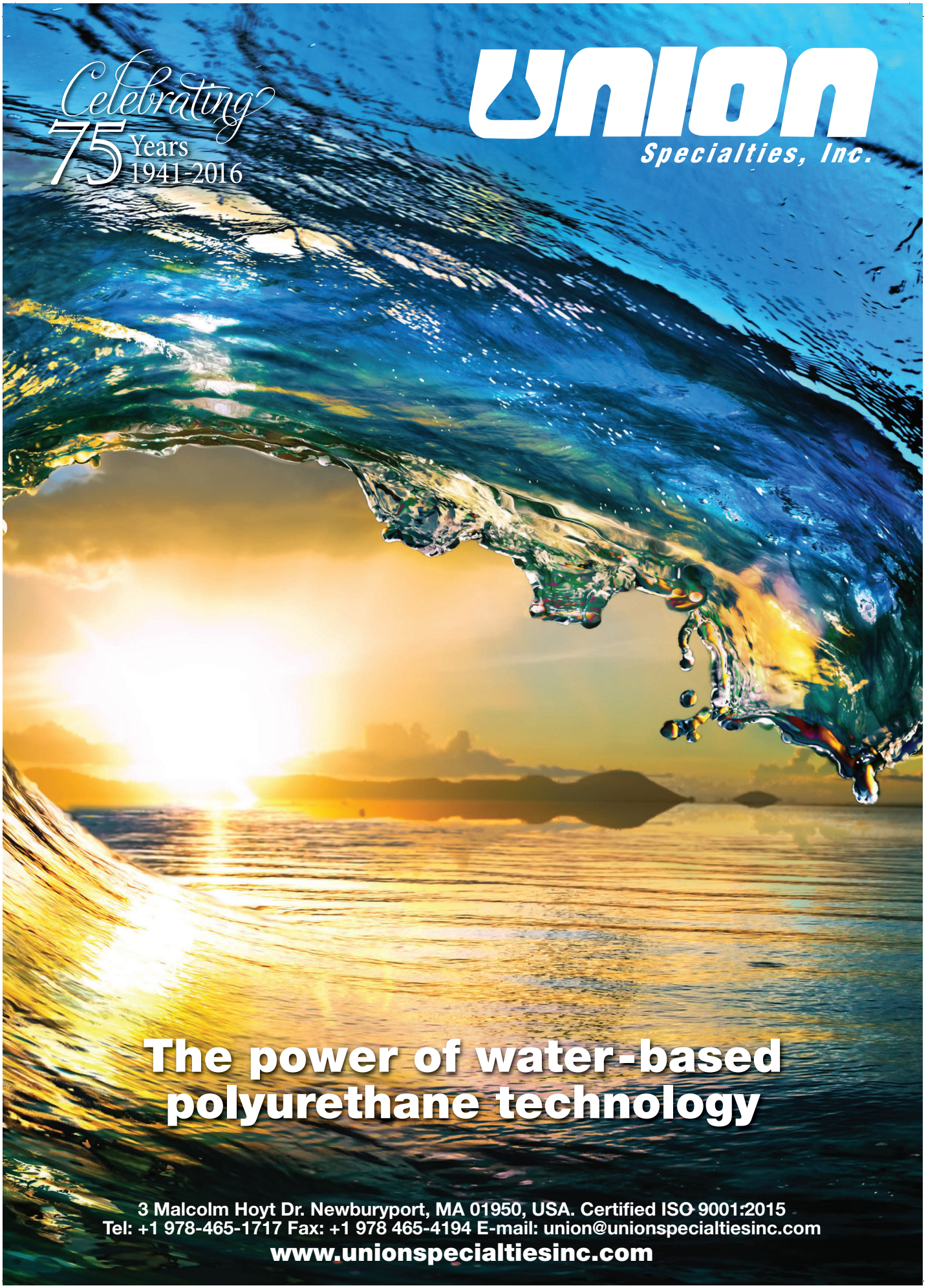
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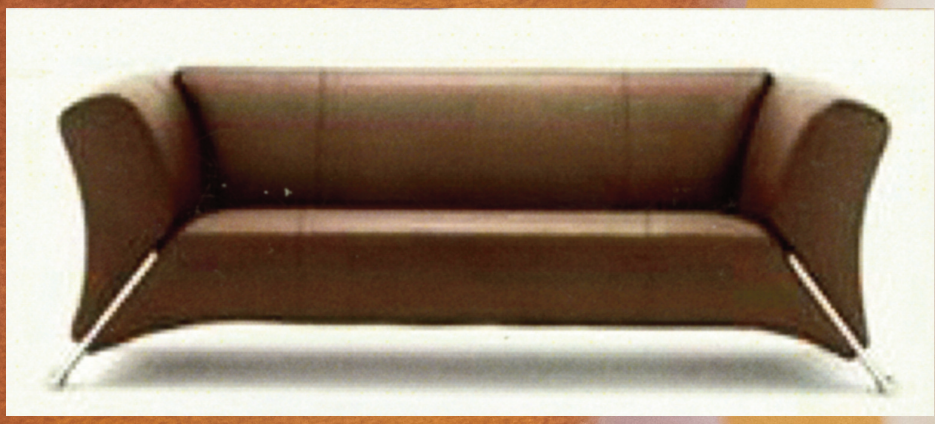

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