Optimization of Dehairing/Fiber Opening using Ammonium Chloride/Dispase by Response Surface Methodology

by

Chao Tang,[1] Hui Liu,[1] Yadi Hu,[1] Fang Wang,[1*] Keyong Tang[1*] and Jide Zhong[2]

[1]*Henan International Joint Laboratory of Biomass Resources and Materials, School of Materials Science and Engineering, Zhengzhou University,* Zhengzhou 450001, China

[2]Henan Prosper Skins & Leather Enterprise Co., Ltd, Mengzhou 454750, China

Abstract

A great number of pollutants including sulfides, suspended solids, and ammonia nitrogen compounds are generated by dehairing and fiber opening with the traditional use of sodium sulfide, calcium hydroxide, and ammonium salt. In this work, ammonium chloride (NH_4Cl)/dispase system was used for the dehairing and fiber opening as an alternative of sodium sulfide/lime system to simplify the beamhouse operations, as well as to ease environmental pollution. It was found that the removing effect of interfibrillar substance is highly related to the factors such as enzyme catalysis time, temperature and the salt usage. Therefore, the mathematical models of enzyme activity, protein content, polysaccharide content and hydroxyproline content were established by response surface methodology (RSM) with the enzyme catalysis time, temperature and the salt usage as independent variables. It was found that the dehairing/fiber opening effect is the best with 2 % NH_4Cl (base on hide weight) at 32.5°C for 15h, and only 3.5 h is needed for dehairing. This work might provide a reference for green leather making with enzymes.

Introduction

Leather making is generally divided into three sections of beamhouse, tanning and finishing.[1] In the beamhouse, the hair and non-collagen components are removed, with the collagen fiber opened for the subsequent tanning. The quality and performance of the resultant leathers are determined by the beamhouse to some degree. Especially, dehairing and liming in beamhouse play an important role in removing the non-collagen ingredients in hides/skins such as soluble protein, elastin and proteoglycan to open the collagen fiber bundles.[2] However, 60%~70% of the total pollution load of the leather industry is produced by dehairing and liming due to the excessive use of lime, sulfide and other chemicals.[3] Among them, 40% of the sulfide does not participate in the reaction, which is directly discharged, resulting in the pollution of sulfide. The sulfide in the wastewater can be easily converted into the toxic gas of H_2S.[4-6] In addition, the discharged wastewater containing high concentrations of sulfide might contaminate the rivers or land, and destroy plants and organisms. Therefore, great effort has been done to remove or recover the sulfide from wastewater containing lime, in order to find effective ways to reduce pollution.

Nowadays, new liming materials such as enzymes have been developed to replace the traditional materials, which have been studied in the dehairing and fiber opening because of the high efficiency, environmental friendliness, low dosage and good specificity. Saravanabhavan et al. used complex enzymes as an alternative of chemical reagents in leather making to reduce environmental pollution.[7] Madhumathi et al. explored the influence of pH, substrate concentration and temperature on enzyme activity.[8] Xu et al. used enzymes for enzymatic dehairing and dispersing collagen fiber bundles, and used silica to adsorb and fix chromium ions.[2] Although some works have been done in the production of leather with enzymes, many problems are still faced in the application of enzymes, such as the shortages of high cost, which limits in the commercial applications. As a result, enzymes are usually used as assistant reagents for dehairing and fiber opening, with sulfide and calcium hydroxide the main reagents. In addition, although proteolytic enzymes could attack the proteoglycans of the hair roots, the collagen in the grain layer may be partially destroyed, resulting in poor quality and poor appearance of the final leather, which should account for the seldom applications in industrial scale. Therefore, how to improve the efficiency of enzyme dehairing and opening efficiency of collagen fiber bundles is critical for the quality of leather and green leather manufacturing.

The response surface methodology (RSM) is an effective mathematical and statistical method to design experiments, build models and search for optimal conditions because less time and resources are needed with easier operation and higher dependability.[9, 10] Zhang et al. investigated different ions in assisting neutral protease for interfibrillar substance removal and collagen fiber bundle opening, and found that NH_4Cl was more effective and eco-friendly.[11] However, there is no study about the effect of NH_4Cl concentration on the dehairing and collagen fiber opening of cowhide. In order to determine the variable range of Box-Behnken design (BBD), the classic one-time variable method was used to study the effects of NH_4Cl concentration, processing time, and processing temperature on the dehairing and collagen fiber opening effect of cowhide. The regression model was established by using the BBD method. Through

* Corresponding author email: cattwm@zzu.edu.cn; kytangzzu@hotmail.com

Manuscript received November 15, 2021, accepted for publication February 2, 2022.

the combination of experiment and RSM simulation, the influence of variables on the process of dehairing of cowhide and collagen fiber opening was studied and analyzed. The results of optimization for dehairing and fiber opening were compared with those of traditional process, which might provide useful information for more efficient application of NH_4Cl/dispase to reduce environmental pollution and improve the quality of leather. In this work, the dehairing effect of cowhides is highly dependent on dehairing time, temperature and salt concentration by the study of control variables. In order to improve the dehairing and fiber opening effects of enzyme, the application condition of enzyme was studied and optimized by response surface methodology (RSM).

Materials and Methods

Materials

NH_4Cl (ACS pure) and sodium sulfide were purchased from Fengchuan Chemical Reagent Technology Co., Ltd, Tianjin, China. Silver nitrate (AR pure) were from Aladdin Reagent Co. Ltd., Shanghai, China. Wet salted cowhides were from Henan Prosper Skins & Leather Enterprise Co., Ltd, Henan, China. Dispase (neutral protease, extracted from Bacillus subtilis by liquid fermentation) and tannin were from Macklin Reagent Co., Ltd., Shanghai, China. Verhoeff's Van Gieson (EVG) dye liquor was provided from Wuhan Servicebio Technology Co., Ltd., Wuhan, China. Distilled water was used in this study.

The treatment of cowhide

A whole wet salted cowhide was cut into five pieces (from head to tail), and one with uniform thickness was put in the drum for washing and soaking. The water was changed every four hours until no precipitate appeared after silver nitrate solution was dropped into the water, meaning that the salt in the hides was completely removed.[12]

The chosen cowhide was cut into 50 mm × 30 mm pieces. In order to investigate the relationship between the NH_4Cl concentration and the dispersion of collagen fiber, seven pieces of cowhide were put in 20 mL salt/enzyme solution with 0.8 wt % dispase and 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 wt % NH_4Cl, respectively, followed by vibration for 5 h. In order to investigate the relationship between the duration and degree of collagen fiber opening, six pieces of cowhide were processed with 1 wt % NH_4Cl and 0.8 wt % dispase at 30°C for 5 h, 10 h, 15 h, 20 h, 25 h, and 30 h, respectively. Finally, the effect of temperature was respectively studied at various temperature of 20, 25, 30, 35, 40, 45, and 50°C, with the 0.8 wt % dispase and 1 wt % NH_4Cl.

EVG staining

The dehaired cowhide samples were embedded by paraffin and cut into slices of 5 mm thickness by a tissue slicer (Shanghai Leica Instrument Co., Ltd., RM2016). After being de-waxed in xylene and ethanol solutions, the slices were stained with EVG (Elastin van Gieson) solution for 5 minutes and then rinsed with water. To improve the differentiation, the slices were re-dyed and sealed with neutral gum. The samples were observed by a digital slice scanning system (Pannoramic 250/ MIDI, Hungary).

Analysis of SEM

The cowhide samples after dispase/NH_4Cl process were dried by vacuum freeze drier (Ningbo Xinzhi Biotechnology Co., Ltd., SCIENTZ-18ND) and then cut into pieces of 4 × 4 millimeter. The surfaces of cowhide samples were sprayed with gold powder for more than 1 min. The pores of cowhide surface were observed by focused ion beam scanning electron microscopy (SEM, S4800, Hitachi, Japan) at 10 kV.

Tanning

The leather samples treated with traditional method or with dispase/NH_4Cl system were soaked in 7 wt % sodium chloride solution with 100 wt % water for 1 hour, then the 4 wt % sulfuric acid which were diluted 10 times were added. After 4 hours, with no float, the sodium thiosulfate was added to be uniformly distributed on the surface of leather sample by vibration (200r/min) for 30 minutes. Water at 300wt% was added at 30°C, and 5 wt %, 10 wt%, 10 wt %, and 10 wt % tannin were used each day. The leather samples were washed in 200wt% water and air-dried at 28°C for 48 hours.

Establishment of RSM model for collagen fiber opening

RSM is a technique that combines specific mathematical and statistical methods. Since response values and variables are correlated with each other, in order to investigate the relationship between responses and variables, a series of experiments can be designed and the experimental results can be used as responses. So RSM models can be established and variables can be optimized.[9]

The advantage of BBD Design in Design Expert software (version 8.0.6) is that each factor has only three levels, which could greatly reduce the experimental workload. Three important parameters including the time (A), the temperature (B), and the NH_4Cl concentration (C) were analyzed through this method, as shown in Table I. According to this method, 17 experiments can be designed to analyze the interaction between three important

Table I
Design factor range and levels (coded)

Independent variable	Units	Coded levels		
		-1	0	+1
Concentration	wt%	0	1	2
Temperature	°C	25	32.5	40
Time	h	5	15	25

influencing parameters,[13] with actual value to each responses shown in Table II.

Analysis of variance was used to determine the influence of factors on experimental results, and the responses obtained by each experiment form response surfaces and contour lines, so that the predicted responses can be visualized more intuitively. The response function for predicting optimal conditions was expressed according to equation (1). The value of R^2 (determination coefficient) was used to evaluate the fitness of the model and the F test was used to check the significance of the model equation. The higher F value means the more accurate model equation.

$$Y = \beta_0 + \Sigma_{i=1}^{n} \beta_i x_i + \Sigma_{i=1}^{n} \beta_{ii} x_i^2 + \Sigma_{i=1}^{n} \beta_{ij} x_i x_j + \cdots + e \qquad (1)$$

Where Y is the result of the dispase activity, proteoglycan concentration, protein concentration, or tensile strength of 17 experiments, β_0, β_i, β_{ii}, and β_{ij} are intercept, linear coefficient, quadratic coefficient, and interaction coefficients, respectively, n is the number of independent variables, X is the independent factors, and e is the random error.[14] Response surfaces and contour plots were obtained while variable of the second-order polynomial model was constant.[15]

Results and Discussion

Effect of NH_4Cl concentration, time, and temperature on collagen fiber opening

NH_4Cl is an ionic compound, soluble in water to form the ions of NH_4^+, Cl^-, OH^- and H^+. The flexibility of dispase can be increased by the interaction with NH_4Cl. The ammonium ion will combine with the negatively charged group of the enzyme to improve the bonding ability of enzyme with substrate and increase the activity of the enzyme. The ionic bonds of interfibrillar substance among the collagen fibers should be destroyed by the strong ionic electrostatic attraction of salt solution, resulting in the hydrolysis of proteoglycan (interfibrillar substance). Therefore, the concentration of NH_4Cl is very important to cooperate with dispase for the dehairing of the cowhide and the opening of collagen fiber. As shown in Figure 1, the activity of dispase increases with the increase of the NH_4Cl concentration until 1.5 wt % because of the salting-in effect of protein to improve the flexibility of the enzyme, while the effect of salting-out might be carried out with the increase of concentration of NH_4Cl. As shown in Figure 1(C), the protein concentration increased and then decreased with the increase of concentration of NH_4Cl due to the change tendency of enzyme activity, as well as the collagen fiber opening degree and the carbohydrate content in wastewater with same tendency.

As shown in Figure 2, the polysaccharides and elastic fiber in cowhides are gradually reduced with increasing the concentration of NH_4Cl (Figures 2(B) and (C)), while high concentration of NH_4Cl is not good for hydrolyzing the polysaccharides and elastic fiber, probably because of the salting-out of enzyme (Figure 2(E)). The enzyme with different concentrations of NH_4Cl was used for dehairing and collagen fiber bundle opening, with the SEM results shown in Figure 3(A, B, C). The pores in the cowhides surface become clearer and clearer with increasing the NH_4Cl concentration, with the pores hydrolyzed at the NH_4Cl concentration of about 2%. The SEM results of leather tanned with tannins were shown in Figure 3 (a, b, c), where the pores on the cowhides surface became clearer with the NH_4Cl concentration increased to 1%.

The effect of temperature on the cowhide dehairing/collagen fiber opening was investigated, with the results shown in Figure 4. The enzyme activity increases with the increase of temperature from 20°C to 50°C, which was probably because of the increase in the thermal movement and permeation of ammonium ions with the increase of temperature, further improving the flexibility of the enzyme to expedite the hydrolysis of glycoproteins and proteoglycans in cowhide.

The effect of time on the depilation/collagen fiber opening was investigated, with the results shown in Figure 5. The tendency of tensile strength of the tanned leather is slightly decreased with the increase of time because of the excessive hydrolysis of collagen fiber with the long time soaking in NH_4Cl/dispase solution.

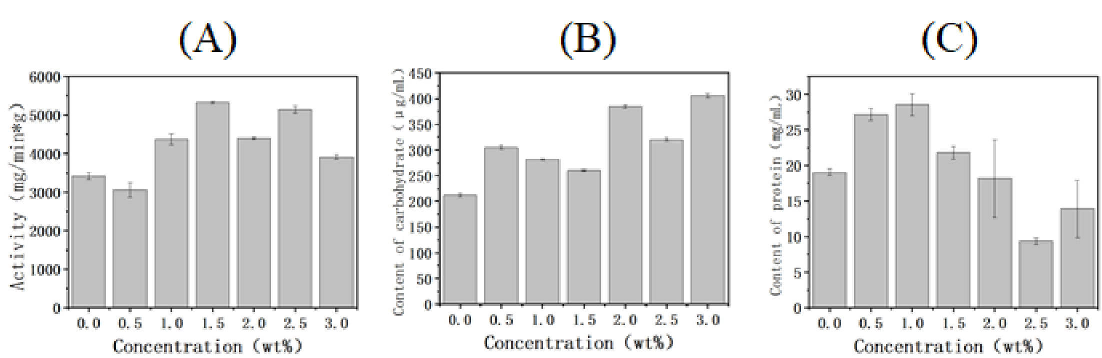

Figure 1. Effect of NH_4Cl concentration on the activity of dispase **(A)**, content of carbohydrate **(B)** and content of protein **(C)**.

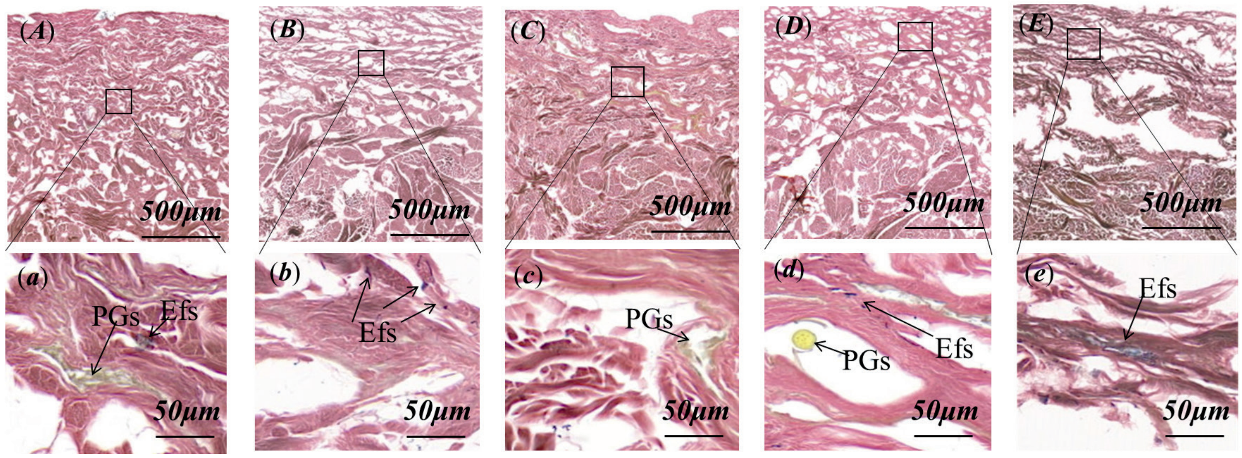

Figure 2. The cross-section images of cowhide processed with 0.8wt% dispase in different concentrations of NH_4Cl by EVG staining: **(A)** 0%, **(B)** 1%, **(C)** 2%, **(D)** 3%, **(E)** 4%.

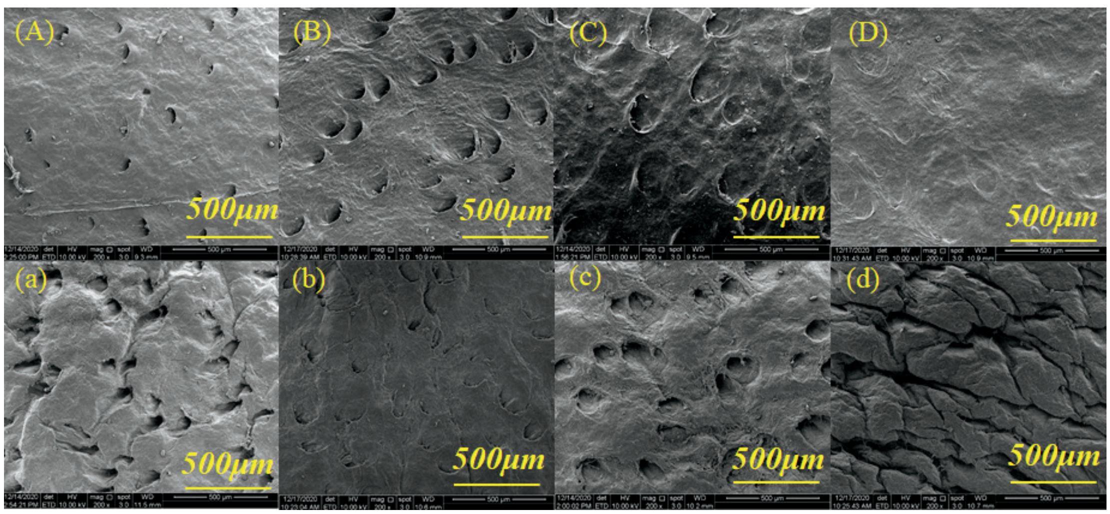

Figure 3. SEM images of surface of samples before (**(A)** 0%, **(B)** 1%, **(C)** 2%, **(D)** 3%) and after (**(a)** 0%, **(b)** 1%, **(c)** 2%, **(d)** 3%) tanning.*

*The usage of dispase was 0.8 wt % and the percentage was the concentrations of NH_4Cl

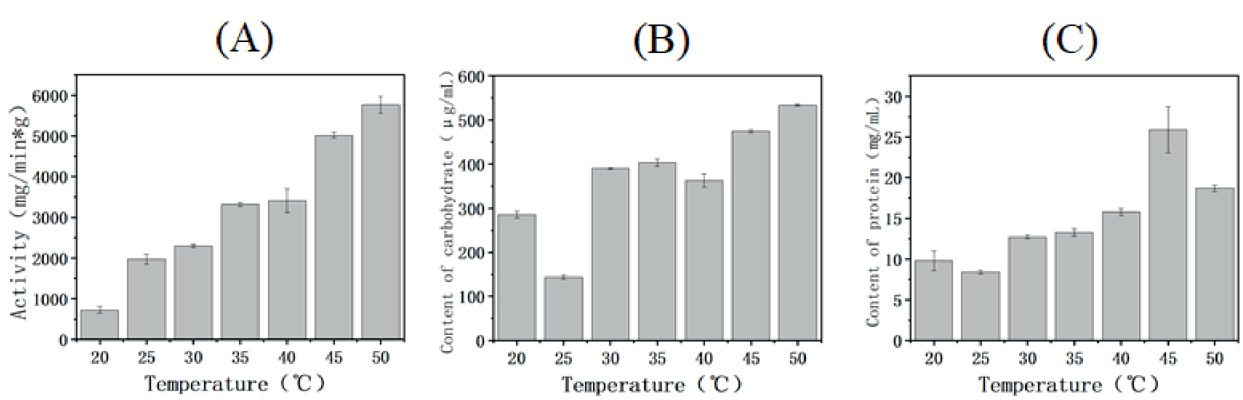

Figure 4. Effect of temperature on the activity of dispase **(A)**, content of carbohydrate in NH_4Cl/dispase solution **(B)**, content of protein in NH_4Cl/dispase solution **(C)**.

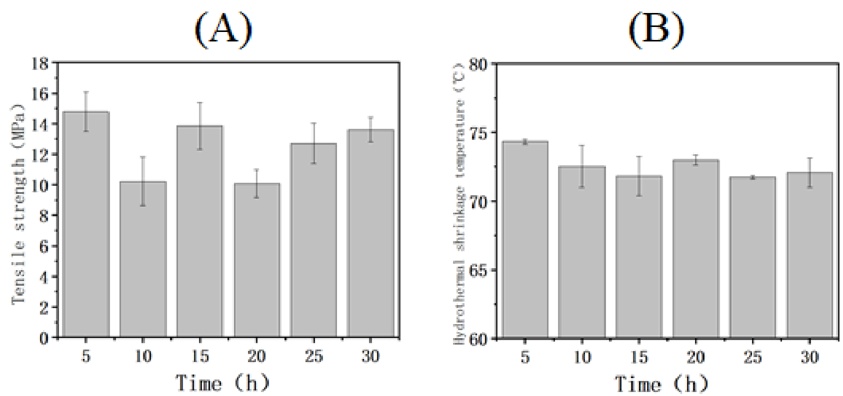

Figure 5. Effect of time on the tensile strength **(A)**, hydrothermal shrinkage temperature **(B)** of tanned leather.

RSM modelling

In the RSM model, the independent variables were converted into coded variables (Table I). In RSM, natural variables were converted into coded variables, these coded variables were defined as dimensionless, which had a mean value of zero, and had the same spread or standard deviation.[13] The results of the dispase activity (Y), proteoglycan concentration (G), protein concentration (H), and tensile strength (K) of 17 experiments were shown in Table II. The response values and independent variables were analyzed by regression of Box-Behnken and a quadratic regression model was proposed.

Corresponding coefficients of multiple determination in the ANOVA of each response for evaluating the effect of dehairing/collagen fiber opening, with the detailed results were shown in Table IV. A maximum value for each model was obtained from ten regression coefficients (Intercept, A, B, C, AB, AC, BC, A^2, B^2, C^2) of the model (Table IV). In general, a fitted response surface may produce poor or misleading results unless the model exhibits an adequate fit during the exploring and optimizing proceeding,[13] which makes the checking of model adequacy essential (Table V). A plot of experimental and theoretical values indicated a normal fit ($R \geq 0.83$, $p < 0.05$) for enzyme activity, content of protein, and tensile strength of tanned leather. A high proportion of variability was explained by the RSM models for evaluating the effect of dehairing/collagen fiber opening. The regression models were significant ($p < 0.02$ or $p < 0.05$) for activity of dispase, content of protein and tensile strength of tanned leather, with general satisfactory coefficient of determination (R^2) varying from 0.83 to 0.86, and there is a high correlation between the predicted values and the actual values, as shown in Figure 6. Moreover, coefficient of variation (CV) describes

Table II

Experimental results of RSM Box-Behnken design

Run	Time h	Temperature °C	NH_4Cl concentration wt%	Activity Y mg/min*g	Content of carbohydrate G μg/mL	Content of protein H mg/mL	Tensile strength K MPa
1	15.00	32.50	1.00	1319.19	15.03	22.98	23.22
2	25.00	32.50	0.00	1056.16	14.14	25.92	22.58
3	5.00	25.00	1.00	1275.08	9.13	13.09	24.29
4	15.00	40.00	0.00	843.06	15.35	30.57	17.80
5	15.00	32.50	1.00	1282.00	11.61	20.11	17.61
6	25.00	40.00	1.00	706.04	9.27	28.63	24.51
7	25.00	25.00	1.00	913.64	7.50	22.16	16.55
8	15.00	40.00	2.00	906.85	20.36	29.96	20.52
9	25.00	32.50	2.00	1754.70	17.22	26.05	15.30
10	5.00	40.00	1.00	1338.04	18.51	20.50	14.74
11	15.00	32.50	1.00	1150.32	11.47	23.70	18.54
12	15.00	32.50	1.00	1208.73	10.22	20.46	22.00
13	5.00	32.50	2.00	1961.57	11.03	19.72	19.38
14	15.00	25.00	2.00	965.34	8.13	18.31	15.02
15	15.00	25.00	0.00	856.12	9.76	19.09	18.2
16	5.00	32.50	0.00	1221.79	9.44	18.08	15.02
17	15.00	32.50	1.00	1127.65	12.77	25.85	20.37

the extent of the dispersed data. The CV for each response value was within the acceptable range (0%~30%). Since CV is a measure expressing standard deviation, the smaller value of CV means that the model has better reproducibility. In general, a high CV indicates that the variation in the mean value is high and does not satisfactorily develop an adequate response model.[13]

An ANOVA of independent variables in Table III indicated that the concentration of NH_4Cl is the most significant ($p < 0.02$ or $p < 0.05$) factor affecting the enzyme activity and tensile strength of all response values. The model suggested that the temperature significantly affects the contents of carbohydrate and protein (Table IV). Thus, NH_4Cl concentration and temperature contribute significantly to the response. On the other hand, the processing time has little effect on responses. By considering the regression coefficients obtained from independent and dependent variables, NH_4Cl concentration and temperature might be the most important factors significantly affecting the quality of the resultant leather.

Table III

ANOVA results for response surface quadratic model

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	Status
Model-activity	1.383E+006	9	1.537E+005	3.90	0.0433	significant
A-time (h)	2.332E+005	1	2.332E+005	5.91	0.0453	
B-temperature (°C)	5842.26	1	5842.26	0.15	0.7118	
C-concentration (%)	3.245E+005	1	3.245E+005	8.23	0.0241	
Model-Content of carbohydrate	113.01	3	37.67	4.21	0.0275	significant
A-time (h)	5.000E-005	1	5.000E-005	5.590E-006	0.9981	
B-temperature (°C)	104.91	1	104.91	11.73	0.0045	
C-concentration (%)	8.10	1	8.10	0.91	0.3586	
Model- Content of protein	294.25	3	98.08	21.30	< 0.0001	significant
A-time (h)	123.01	1	123.01	26.71	0.0002	
B-temperature (°C)	171.22	1	171.22	37.18	< 0.0001	
C-concentration (%)	0.018	1	0.018	3.920E-003	0.9510	
Model-tensile strength	141.58	9	15.73	5.08	0.0217	significant
A-time (h)	3.24	1	3.24	1.05	0.3403	
B-temperature (°C)	0.19	1	0.19	0.060	0.8136	
C-concentration (%)	7.70	1	7.70	2.49	0.1587	

Table IV

Regression coefficients of predicted quadratic polynomial models for the activity of dispase, content of carbohydrate and content of protein of NH_4Cl/dispase solution, and tensile strength of tanned leather

Coefficient	Activity of dispase Y mg/min*g	Content of carbohydrate G μ g/mL	Content of protein H mg/mL	Tensile strength K MPa
Intercept	1217.58	12.41	22.66	15.90
A-time(h)	-170.74	2.500E-003	3.92	-0.64
B-temperature (°C)	-27.02	3.62	4.63	0.15
C-concentration (%)	201.42	1.01	0.047	0.98
AB	-67.64			-1.14
AC	-10.31			0.10
BC	-11.36			-0.81
A^2	223.17			4.95
B^2	-382.55			1.77
C^2	57.81			0.31
R-Squared	0.8336	0.4929	0.8309	0.8672
C.V. %	16.98	24.10	9.47	9.16

Table V

ANOVA results for response surface quadratic model

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	Status
Activity of dispase mg/min*g						
Residual	2.762E+005	7	39450.56			
Lack of Fit	2.490E+005	3	82996.58	12.22	0.0175	significant
Pure Error	27164.16	4	6791.04			
Cor Total	1.659E+006	16				
Content of carbohydrate μ g/mL						
Residual	116.28	13	8.94			
Lack of Fit	103.15	9	11.46	3.49	0.1205	not significant
Pure Error	13.13	4	3.28			
Cor Total	229.29	16				
Content of protein mg/mL						
Residual	59.86	13	4.60			
Lack of Fit	37.17	9	4.13	0.73	0.6831	not significant
Pure Error	22.69	4	5.67			
Cor Total	354.11	16				
Tensile strength MPa						
Residual	21.67	7	3.10			
Lack of Fit	18.25	3	6.08	7.11	0.0442	significant
Pure Error	3.42	4	0.86			
Cor Total	163.26	16				

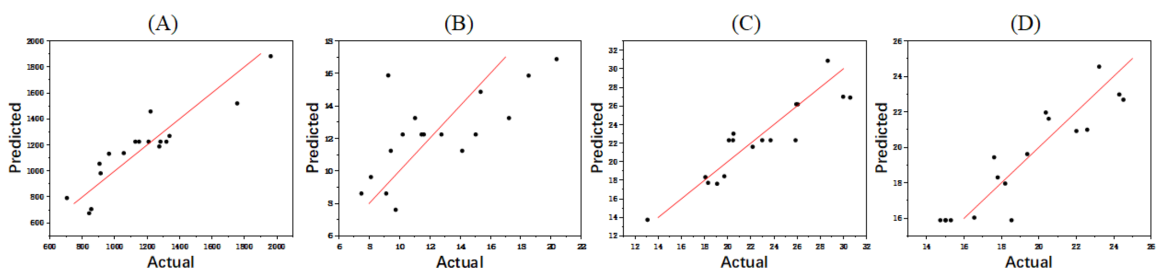

Figure 6. Comparison of predicted and experimental responses of RSM results: **(A)** activity of dispase, **(B)** content of carbohydrate, **(C)** content of protein, **(D)** tensile strength of tanned leather.

Analysis of response surfaces

As shown in Figures 7~10, three-dimensional (3D) response surface and two-dimensional (2D) contour plots were used to explain the effects of time, temperature and NH_4Cl concentration on the dispase activity, content of carbohydrate, content of protein, and tensile strength, respectively. Figure 7 (a) ~ (c) showed the effects of temperature, NH_4Cl concentration and time on the enzyme activity. Dispase activity decreases with increasing the time, while dispase activity firstly increases with the temperature increasing until 32°C, and then decreases. As the concentration of NH_4Cl increases, the activity of the dispase increases. The two-dimensional contour map has greater density and greater influence. In Figure 7 (d), compared with the process temperature, the contour density of the process temperature is greater. The process temperature greatly affects the dispase activity. In addition, from Figure 7 (e) (f), we know that the contour density of the NH_4Cl concentration is greater than the process time and process temperature. Therefore, the NH_4Cl concentration has a greater influence on the dispase activity than the process time.

By the use of NH_4Cl/dispase system, the collagen fiber bundles could be opened. The increase of the gap between the fiber bundles is accompanied with the disappearing of the proteoglycan in them. With increasing the temperature, the concentration of proteoglycan of the solution firstly increases to reach a maximum at 32°C. As the NH_4Cl concentration increases, the polysaccharide concentration increases too. The concentration of proteoglycan in the solution does not increase significantly with increasing treatment time,

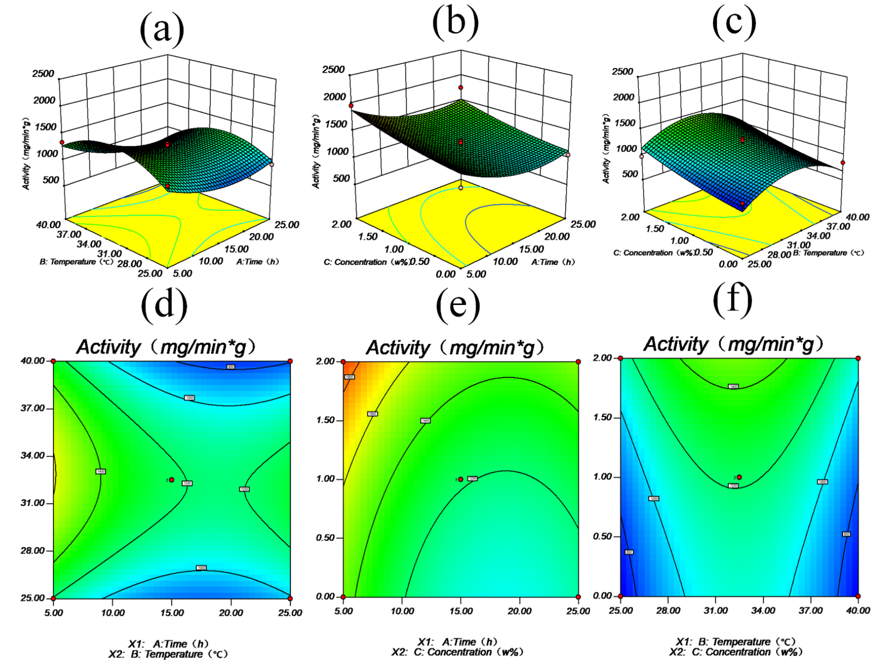

Figure 7. Three-dimensional response plots and two-dimensional contour plots showing **(a) (d)** the effect of time (x1), temperature (x2), and their mutual interaction on dispase activity (Y); **(b) (e)** the effect of time (x1), NH_4Cl concentration (x2) and their mutual interaction on Y; **(c) (f)** the effect of temperature (x1), NH_4Cl concentration (x2) and their mutual interaction on Y.

probably because of the reaction and adhesion between the groups of polysaccharides and the collagen fiber after the polysaccharide being shed.

In Figure 8 (d), compared with the treatment time, the contour density of the treatment temperature is greater. So, the treatment temperature has a greater impact on the proteoglycan removal. Besides, in Figure 8 (e), the contour density of the NH_4Cl concentration is greater than the treatment time. Therefore, the NH_4Cl concentration has a greater influence on the shedding of proteoglycans than the treatment time.

There is glycoprotein among the collagen fiber bundles in hides. The shedding of glycoproteins might increase the fiber bundle gaps. At the same time, dispase will also decompose the surface of collagen to increase the protein concentration in the solution. In Figure 9 (a), with the temperature increasing, the protein concentration of the solution increases. The protein concentration

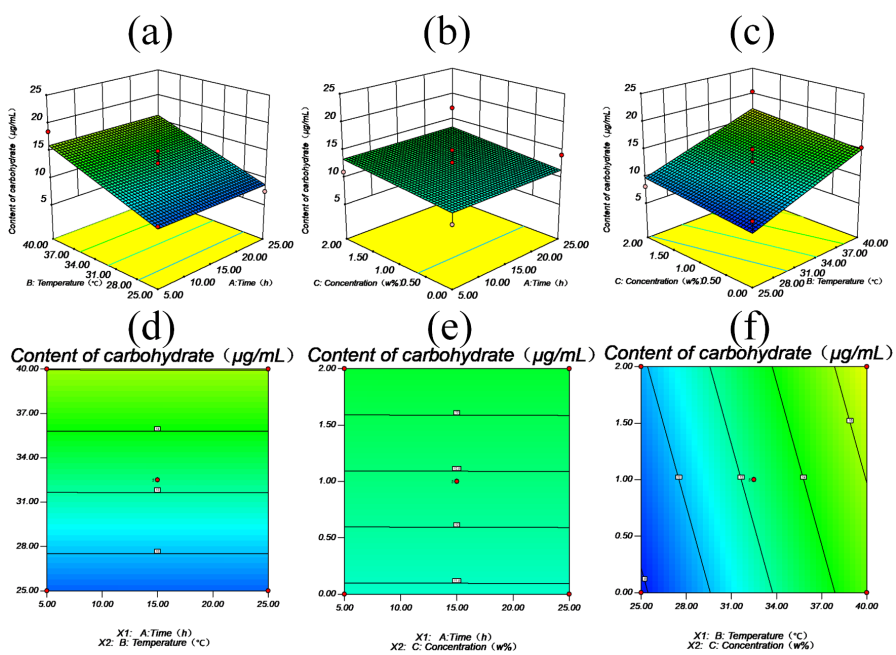

Figure 8. Three-dimensional response plots and two-dimensional contour plots showing **(a) (d)** the effect of time (x1), temperature (x2), and their mutual interaction on content of carbohydrate (G); **(b) (e)** the effect of time (x1), NH_4Cl concentration (x2) and their mutual interaction on Y; **(c) (f)** the effect of temperature(x1), NH_4Cl concentration (x2) and their mutual interaction on G.

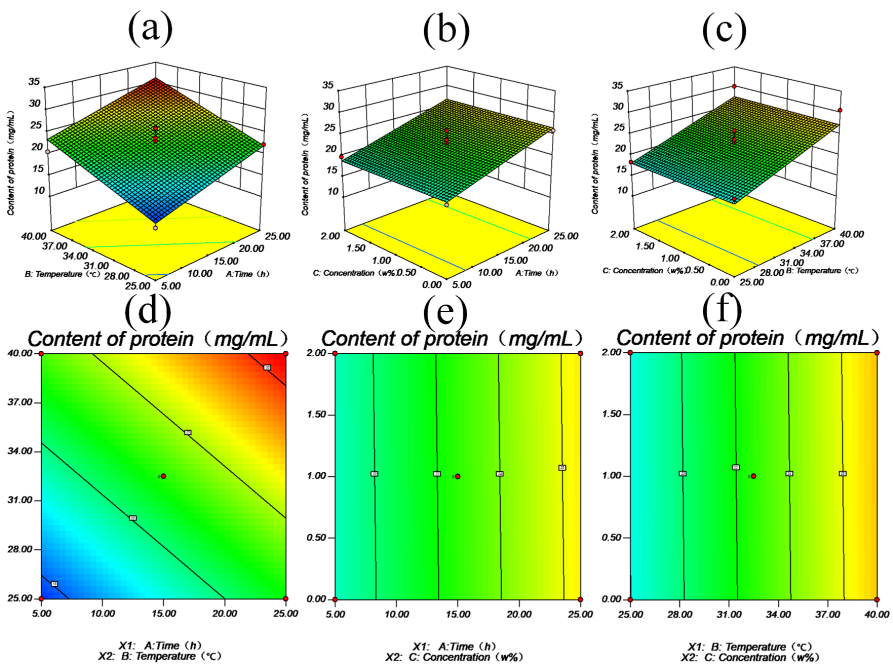

Figure 9. Three-dimensional response plots and two-dimensional contour plots showing **(a) (d)** the effect of time (x1), temperature (x2), and their mutual interaction on content of protein (H); **(b) (e)** the effect of time (x1), NH_4Cl concentration (x2) and their mutual interaction on Y; **(c) (f)** the effect of temperature (x1), NH_4Cl concentration (x2) and their mutual interaction on H.

of the solution is not significantly increased with increasing treatment time. In Figure 9(b) and Figure 9(c), with increasing the concentration of NH_4Cl, the concentration of protein in the solution increases slightly. As shown in Figure 9(d), Figure 9(e), and Figure 9(f), the contour of temperature is larger than the contour density of NH_4Cl concentration and treatment time. Therefore, the influence of temperature on the shedding of glycoprotein is greater than the NH_4Cl concentration and process time.

The dispersion effect of collagen fiber will affect the tanning effect. With increasing the gaps between the collagen fiber bundles, more possibility for the cross-linking between collagen fiber and tannin is provided, resulting in better tanning effect. In Figure 10 (a), with increasing the temperature and treatment time, the tensile strength of the tanned leather firstly decreases and then increases, which should be because of the larger gap between collagen fiber became larger and higher degree of dispersion. After 15h, the degree of dispersion increases. The

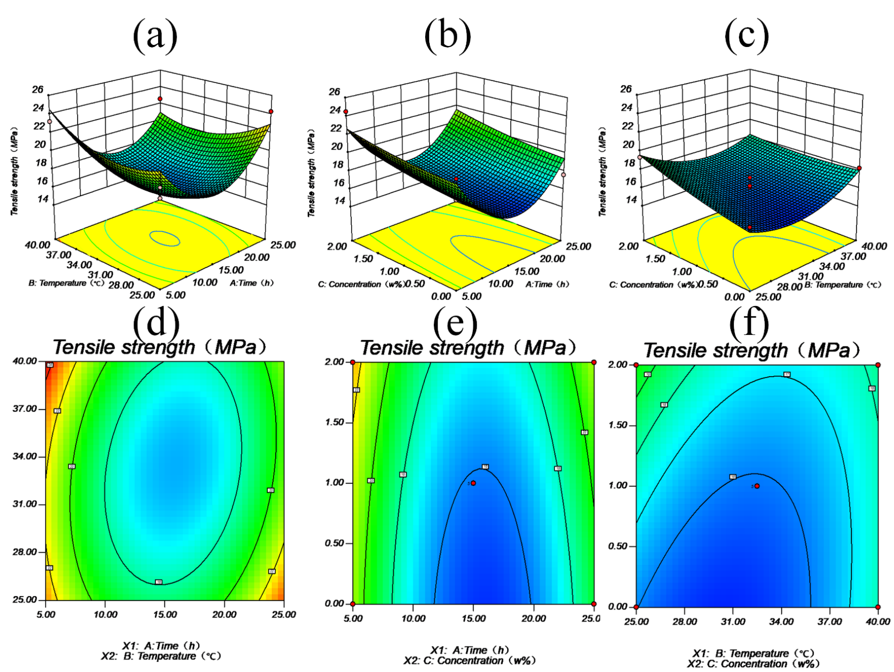

Figure 10. Three-dimensional response plots and two-dimensional contour plots showing **(a) (d)** the effect of time (x1), temperature (x2), and their mutual interaction on tensile strength (K); **(b) (e)** the effect of time (x1), NH_4Cl concentration (x2) and their mutual interaction on Y; **(c) (f)** the effect of temperature (x1), NH_4Cl concentration (x2) and their mutual interaction on K.

Table VI

Pollution loads in waste-water by different processes (unit: kg/t of raw hide)

		Process(kg/t)			Total(kg/t)
			Fiber opening		
		Dehairing	Reliming	Deliming	
C	COD	1.51±0.11	1.05±0.20	0.29±0.42	2.85±0.53
	NH_3-N			0.35±0.10	0.35±0.10
	Cl-	6.33±0.22	0.85±0.11	4.72±0.13	11.90±0.40
	TS	35.75±0.00	8.30±0.00	7.45±0.00	51.50±0.00
NH_4Cl/D	COD		0.37±0.10		0.37±0.10
	NH_3-N		0.20±0.10		0.20±0.10
	Cl^-		6.77±0.02		6.77±0.02
	TS		18.40±0.00		18.40±0.00

(Note:D:dispase)

increase of the cross-linking degree of collagen fiber by tannins leads to an increased tensile strength. In Figure 10 (b) and Figure 10 (c), as the NH_4Cl concentration increased, the tensile strength increased. In Figure 10 (d), the contour of temperature is greater than the contour density of the treatment time, and the contour of the concentration is greater than the contour density of temperature, which means that the NH_4Cl concentration has a greater influence on fiber opening.

Determination of optimization conditions

By analyzing the influence of NH_4Cl concentration, temperature and treatment time on the fiber opening effect of collagen fiber, it was found that the fiber opening effect of collagen fiber increases with increasing the concentration of NH_4Cl. Considering economic benefits and environmental issues, the optimal NH_4Cl concentration was determined at about 1 wt %. With increasing the temperature

Table VII

Physical properties and hydrothermal shrinkage temperature of tanned leathers by different processes

Sample	Tensile strength (MPa)	Elongation at break (%)	Elastic modulus (MPa)	Hydrothermal shrinkage temperature (°C)
C	10.3±0.5	29.3±3.0	0.3±0.1	71.6±0.2
NH_4Cl/D	13.1±1.1	30.9±4.9	0.5±0.1	71.4±0.3

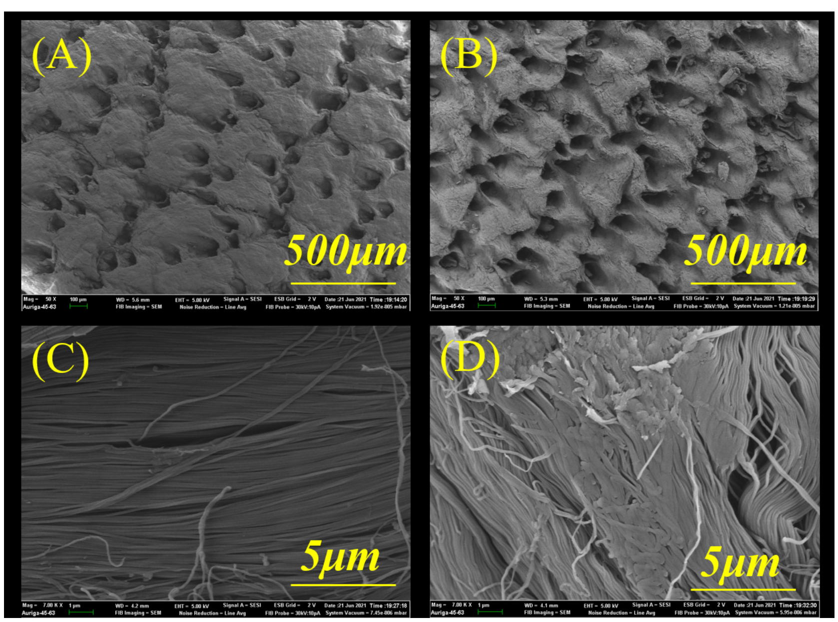

Figure 11. SEM images of the surface of tanned leathers in the predicted optimized condition from NH_4Cl/dispase **(A)** and traditional **(B)** Cross-section SEM images of tanned leather from NH_4Cl/dispase **(C)**and traditional **(D)** processes.

and time, both the enzyme activity and the dispersibility of collagen fiber were significantly increased. However, denaturation of collagen fiber and hydrothermal shrinkage will take place at high temperature. Therefore, the optimized temperature was determined to be 32°C. With increasing the treatment time, the dispersibility of collagen fiber increases. Since enzymes might decompose the protein on the cowhide surface, 15 h is the optimal processing time for fiber opening of collagen fiber.

Comparison of the tanned leathers obtained in optimized conditions and by traditional process

The optimized conditions were obtained through response surface analysis and compared with the traditional process. As shown in Table VI, the chemical oxygen demand (COD), ammonia nitrogen (NH_3-N), chloride ion (Cl^-), and the total solids (TS) of traditional process were significantly higher than those by the optimized NH_4Cl/diapase process, because of the use of sodium sulfide and calcium hydroxide, which caused serious suspended solids and pollution of sulfide and ammonia nitrogen. In addition, the NH_4Cl/diapase system has a good selective catalytic effect on the proteoglycans and elastin in the cowhide.

As shown in Table VII, the tensile strength, elongation at break, and elastic modulus of the samples by the optimized NH_4Cl/dispase dehairing and collagen fiber bundles opening is higher than those by the traditional process, with comparable hydrothermal shrinkage temperature of the tanned leather to the traditional one. Because $Na_2S/Ca(OH)_2$ system is used in the traditional dehairing and liming process, the swelling of the rawhide is reached by the breakage of the hydrogen bonds to loosen the collagen fiber structure, resulting in the decrease in tensile strength, elongation at break and modulus of elasticity of the tanned leather. The NH_4Cl/dispase system might promote the hydrolysis of proteoglycan and elastin to open the collagen fiber bundles with no obvious swelling and more reactive sites for tanning were provided, which was helpful to increase the tensile strength, elongation at break and elasticity modulus of the resultant leathers.

The collagen fiber bundles opening of the sample processed with the optimized NH_4Cl/dispase method was compared with that by the traditional method. The SEM image of the surface of the sample after tanning was shown in Figure 11 (a) (b), where the pores on the grain by NH_4Cl/dispase were more uniform. The SEM image of the longitudinal section after tannin tanning was shown in Figure 11(c) (d), indicating better collagen fiber bundle opening by the NH_4Cl/dispase system, which was conducive to the subsequent process of leather.

Conclusions

The response surface methodology was used to directly analyze the influence of various factors on the process of dehairing and collagen fiber bundle opening. In the NH_4Cl/dispase system, the dehairing effect of cowhide is highly dependent on time, temperature and salt concentration. The dehairing and collagen fiber bundle opening conditions optimized by the response surface methodology are 1 wt % NH_4Cl at 32°C 0.8 wt % dispase solution for 15 h. The physical properties of the tanned leather by the optimized NH_4Cl/dispase system are superior to those by the traditional dehairing method, with the tensile strength increased by 27.18%, elongation at break increased by 5.46%, and elastic modulus increased by 66.67%. The thermal properties of the tanned leather by the optimized NH_4Cl/dispase system are similar to those by the traditional dehairing process. The environmental benefit of the optimized NH_4Cl/dispase system is better than that of the traditional one. The chemical oxygen demand (COD), ammonia nitrogen (NH_3-N), chlorine ion (Cl^-) and total solid (TS) are reduced by 87.02 %, 42.86 %, 43.11 % and 64.27 % respectively. These results indicate that the reasonable use of NH_4Cl can be achieved. By the study, theoretical basis and experimental support might be provided to reduce the environmental pollution as well as improve the dehairing efficiency with comparable or increased leather quality.

Acknowledgments

The financial supports from the National Natural Science Foundation Commission of China (No. 52073262, 51673177) are greatly appreciated.

References

1. P. Thanikaivelan, J.R. Rao, B.U. Nair, T. Ramasami; Progress and recent trends in biotechnological methods for leather processing. *Trends Biotechnol.* **22**, 181-188, 2004.
2. W. Xu, L.F. Hao, Q.F. An, L. Zhou; Minimization of the Environmental Impact of Leather Processing: A Benign and Enzyme-Based Integrated Leather Processing Technology. *Adv. Mater. Res.* **113-116**, 1614-1618, 2010.
3. K. Kolomaznik, M. Adamek, I. Andel, M. Uhlirova; Leather waste--potential threat to human health, and a new technology of its treatment. *J Hazard Mater.* **160**, 514-520, 2008.
4. J. Zhang, W. Chen; A faster and more effective chrome tanning process assisted by microwave. *RSC Advances.* **10**, 23503-23509, 2020.
5. Y.-n. Wang, Y. Zeng, J. Zhou, W. Zhang, X. Liao, B. Shi; An integrated cleaner beamhouse process for minimization of nitrogen pollution in leather manufacture. *J. Clean. Prod.* **112**, 2-8, 2016.

6. R. Zhu, C. Yang, K. Li, R. Yu, G. Liu, B. Peng; A smart high chrome exhaustion and chrome-less tanning system based on chromium (III)-loaded nanoparticles for cleaner leather processing. *J. Clean. Prod.* **277**, 2020.
7. P. Thanikaivelan, C.K. Bharath, S. Saravanabhavan, J.R. Rao, B. Chandrasekaran, N.K. Chandrababu, B.U. Nair; Integrated hair removal and fiber opening process using mixed enzymes. *Clean. Technol. Environ. Policy.* **9**, 61-68, 2006.
8. C.S. Madhumathi M, Saravanabhavan S, et al.; Factors influencing activity of enzymes and their kinetics[J]. *Appl. Biochem. Biotechnol.* 2007.
9. B.S. Mohammed, V.C. Khed, M.F. Nuruddin; Rubbercrete mixture optimization using response surface methodology. *J. Clean. Prod.* **171**, 1605-1621, 2018.
10. M.A. Mujtaba, H.H. Masjuki, M.A. Kalam, H.C. Ong, M. Gul, M. Farooq, M.E.M. Soudagar, W. Ahmed, M.H. Harith, M.N.A.M. Yusoff; Ultrasound-assisted process optimization and tribological characteristics of biodiesel from palm-sesame oil via response surface methodology and extreme learning machine - Cuckoo search. *Renew. Energ.* **158**, 202-214, 2020.
11. Y. Zhang, H. Liu, K. Tang, J. Liu, X. Li; Effect of different ions in assisting protease to open the collagen fiber bundles in leather making. *J. Clean. Prod.* **293**, 2021.
12. H. Liu, Z. Yin, Q. Zhang, X. Li, K. Tang, J. Liu, Y. Pei, X. Zheng, C.E. Ferah; Mathematical modeling of bovine hides swelling behavior by response surface methodology for minimization of sulfide pollution in leather manufacture. *J. Clean. Prod.* **237**, 2019.
13. Y. Yang, Z. Zheng, D. Zhang, X. Zhang; Response surface methodology directed adsorption of chlorate and chlorite onto MIEX resin and study of chemical properties. *Environ. Sci.: Water Res. Technol.* **6**, 2454-2464, 2020.
14. B.D. Yirsaw, M. Megharaj, Z. Chen, R. Naidu; Reduction of hexavalent chromium by green synthesized nano zero valent iron and process optimization using response surface methodology. *Environ. Technol. Innov.* **5**, 136-147, 2016.
15. C. Liyanapathirana, F. Shahidi; Optimization of extraction of phenolic compounds from wheat using response surface methodology. *Food Chem.* **93**, 47-56, 2005.