Studies on Alkaline Protease from *Bacillus crolab* MTCC 5468 for Applications in Leather Making

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Abstract

Bating has become an indispensable operation of leather making to obtain good quality leather. Alkaline proteases are commonly used in leather manufacture in the process of unhairing and bating of hides/skins. In this study, experiments were carried out to test the bating efficacy of a new alkaline protease obtained from *Bacillus crolab* MTCC 5468 and compared with commercial bate. Impact of alkaline protease on porosity of enzymatically and conventionally unhaired goat skins, have been compared. New enzyme treated pelt was free from scud and pigments, with fine, clean, white, silkier grain and pliable. The result was superior to that of commercial bate treated pelts. The pore size/distribution and water vapor permeability was better than commercial bate and conventionally unhaired skins. Scanning electron microscopy and histological analysis of pelt obtained from new alkaline protease based process reveals complete removal of scud and better opening up of collagen fibers. Moreover, the collagen was not damaged and resulted in good quality of leather. The physio-chemical studies conclusively show that among the experiments, the skins unhaired with new alkaline protease was better in comfort properties than the conventionally unhaired skins as inferred by porosity measurements. Using new alkaline protease for bating led to better results than commercial bate. The study indicates improved efficiency of bacterial alkaline protease in leather processing for beam house applications.

Introduction

The leather industry world-wide is under pressure from environmental regulators to comply with stringent discharge legislations. Leather industry contributes significantly to industrial pollution in some region and major pollution causing chemicals viz., sodium sulphide, common salt, lime, solvents etc. are mainly used in large quantities in pre-tanning operations of leather making. To mitigate the pollution and safety issues biological materials like enzymes are being employed increasingly.[1] The global research activity in leather making is shifting towards the design and utilization of cleaner and safer options including enzymatic processes. The production of these enzymes using low cost substrates would reduce the cost of the production and enable economic benefits.[2] Enzymes pave way for bio-processing of hides/skins, offering effective bio-treatments particularly for the unhairing and bating processes.[3]

Enzymes play a significant role in pre-tanning operations. Bating is the treatment of unhaired hides/skins with an enzyme solution to remove inter-fibrillary unwanted proteinaceous constituents. The purpose of bating operation is to effect an internal separation of collagen fibers by degrading keratinous body, to expose a maximum reactive surface for subsequent tanning agents. It allows the removal of scud, a mixture of epidermal matter, hair roots, pigments, fat and glands thereby cleaning the pelt and thus giving the finished leather the desired characteristic properties like feel, softness, stretch, suppleness and pliability.[4,5] Failure to remove the non-collagenous proteins causes cementing together of the fibers when leather is dried and results in firmness and rigidity.

Several bacterial species including *Bacillus subtilis* have been isolated which produces neutral or alkaline proteases suitable for bating operations.[6] Bating in alkaline conditions is universally recognized by the entire leather industry, but to be effective it should be conducted at 35-37°C and at pH 8.0 to 10.0 otherwise the enzyme efficiency drops drastically.[7]

An earlier study involved utilization of the enzyme (Alkaline protease from *Bacillus crolab MTCC 5468)* for sulphide free unhairing.[8] The study also reported the environmental benefits accrued due to such invention. Present study deals with potential changes in leather processing and leather characteristics using the alkaline protease from *Bacillus crolab MTCC 5468* including use as a bate in conventional sulphide based unhairing system and also possibility of eliminating bating operation when alkaline protease is used for unhairing

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The aim of present work deals with the application of crude alkaline protease from *Bacillus crolab* MTCC 5468 as unhairing and/ or bating agent in leather processing and thus replacing the traditional methods and pancreatic proteases with a commercially competitive bating process, and gain economic benefits.

Materials and Methods

Materials

A strain of *Bacillus crolab* MTCC 5468 was used for the production of alkaline protease by solid state fermentation. A single solid substrate (wheat bran) was moistened with distilled water, sterilized and then inoculated with 30% v/w of bacterial pre-culture (MTCC No.5468) and incubated at a temperature in the range of 30-37°C for a period of 96 hrs. At the end of fermentation, the fermented substrate was extracted by homogenization in tenfold volume of 0.2 M carbonate-bicarbonate buffer at pH 9.0 and thus obtained extract was used as a source of crude enzyme (protease). The protease activity of the crude enzyme solution was found to be 38,000 - 42,000U/g of dry substrate and protein content was in the range of 75-92 mg/g of dry substrate. The crude enzyme used in this study is stable over temperature range of 28-45°C and revealed optimum activity in the pH range of 8-12.[9] Thus produced protease enzyme was taken for further studies. Commercial bate for bating experiments were sourced from Tex Bio sciences India Pvt Ltd to serve as control showing protease activity in the range of 2000 – 2200 LVU.

Methods

The bating efficiency of the crude enzyme was evaluated at laboratory scale. Two different sets of experiments were carried out as shown in the table along with two batches of conventional process based trials to serve as control.

Experiments	Unhairing	Bating
Unhairing experiment - I	Enzymatic unhairing MTCC 5468 (Alkaline protease)	No bating
Unhairing control - I	Conventional unhairing (Lime + sulphide)	No bating
Bating experiment - II	Conventional unhairing (Lime + sulphide)	Alkaline protease MTCC 5468
Bating control - II	Conventional unhairing (Lime + sulphide)	Commercial bate

The new alkaline protease has been used as an unhairing agent, avoiding conventional bating (Experiment-I). Conventional lime- sulphide unhairing was followed for two sets of skins, out of which bating was not carried out for one batch (Control-I) and bating was done for other (Control-II). Further, the new alkaline protease was also used as a bate for conventionally unhaired skins (Experiment-II).

Two goat skins were taken and soaked in 300% water for 4 to 5 hrs. In the experiment-I, the unhairing was done by using enzyme 1.2% (w/w), while in the control-I unhairing was carried out using conventional method (sulphide-2.5-3%; lime-8-10%; water- 20%; paste applied on flesh side; temperature-30-35°C). The unhaired skins were delimed, pickled and tanned. Bating step was avoided in both the trials, to check the bating efficacy of the enzymatically unhaired and conventionally unhaired skins.

In the control-II and experiment-II two wet salted goat skins, weighing approximately 1kg each, were used for bating trails. The skins were soaked in 300% water (v/w); unhairing was done by traditional chemical method- 10% lime and 2.5% sodium sulphide. The limed skins were washed thoroughly and delimed with 100% water and 3% ammonium chloride for 40 min and then delimed pelts were subjected to bating process-commercial bate (1%) and alkaline protease (1%) were added to the respective control and experiment. At the end of each experiment, the extent of bating was assessed by air bubble and thumb impression test. The tanned skins were converted into crust leather as per the standard methods and were evaluated for their organoleptic properties.

Qualitative Analysis for Efficiency of Bating

After bating, to perform air-bubble test, the pelt was folded, held tightly and bubble was created. It was gradually made smaller, maintaining the amount of air inside the bubble.[5] This creates pressure within the bubble, forcing from the inside to the outside, but only if transmission is allowed by the degree to which the fiber structure is opened. For thumb impression test, the time taken by the bated pelts to regain its original shape after thumb was pressed on the grain side of the skin and the intensity of the impression was noted.[5] The scud removal was assessed by scraping the finger nails on grain of the pelt.

Analysis of Unhairing and Bating Spent Liquor

The experimental liquor samples were collected and centrifuged at 8000 rpm for 6 min. Then the protein content in supernatant was determined by the method of Lowry *et al.*[10] using bovine serum albumin as a standard

Chrome Liquor Analysis

The chrome content of the experimental leathers was analyzed by standard IUC method [11, 12] to assess their chromium uptake capability. The results were expressed on dry weight basis of respective wet blue leathers.

Water Vapor Permeability Measurement

The standard method of LST ENISO 14268[13] was followed to measure the water vapor permeability. The specimen was placed on mouth of the jar containing solid desiccant, with strong air current under constant agitation and conditioned atmosphere. The mass of moisture passed through the test specimen and that absorbed by the desiccant is determined by weighing the jar. To determine the water vapor absorption, impermeable material and the specimen was clamped over the, which holds water, for time period of the about 8 hrs. Water vapor coefficient was calculated using obtained values of permeability and absorption. Specimen was then weighed and the water absorption was determined by the mass difference before and after the test.

$$\text{Water vapour permeability, mg/cm}^2\text{/hr} = \frac{7639 \times W}{D^2 \times t}$$

Where, 7639 is conversion factor (area and time); W is change in mass of the test port assemblies in mg; D^2 is diameter; t is time

Capillary Flow Porometry Analysis

The pore size and its distribution was analyzed using PMI capillary flow porometer. The experimental wet blue leather samples were conditioned and made in to diameter 20 mm diameter pieces and the thickness was measured.[14] Initially, a non-reacting gas was sent through the test samples. This was followed by wetting with liquid of known surface tension (Calwick, surface tension-15.9 dynes cm^{-1}). The changes in flow rate were measured as a function of pressure for both dry and wet processes. (Pressure vs. gas flow rate) wet and dry profiles of each sample were determined. The pore size was calculated by the software for porous materials using the equation:

$$D = \frac{4\gamma \cos\theta}{P}$$

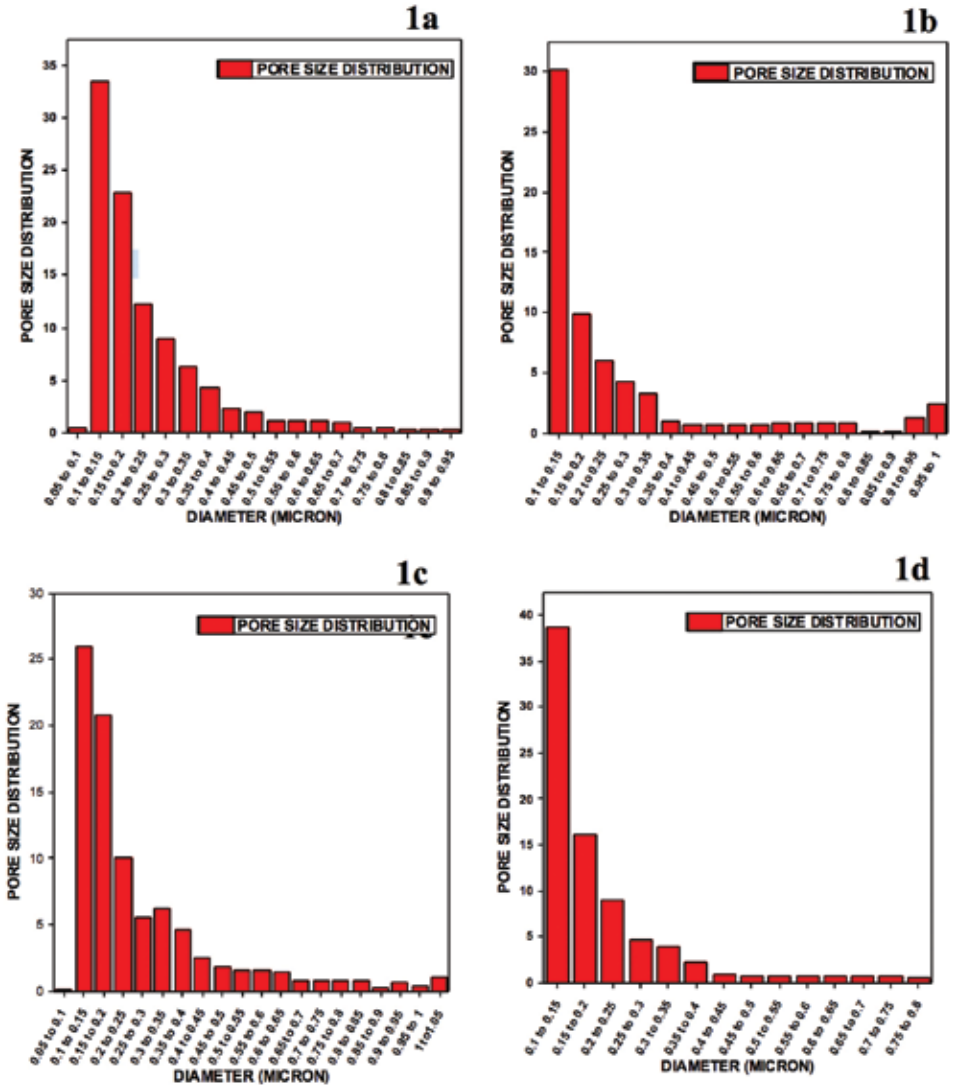

Figure 1. Capillary porometry analysis of control and experimental leather samples showing the pore size and its distribution from Unhairing experiment-1 (1a), Unhairing control-I (1b), Bating experiment-II (1c), Bating control-II (1d).

Where, **D** is pore diameter; **γ** is surface tension of the wetting liquid (15.9 dynes cm^{-1}); **θ** is contact angle of the wetting liquid; **p** is differential pressure.

Histological Analysis

Samples of approximately 5 cm^2 sizes were cut from the sampling portions of bated pelt of all experimental skins. The pelts were prepared for histological analysis using method described by Bancroft *et al.*[15,16] Briefly, the experimental samples were washed thoroughly with water followed by formaldehyde fixation at a concentration of 10% (v/w). The samples were serially dehydrated with 30%, 50%, 70%, 90% and 100% (v/v) of ethyl alcohol followed by xylene treatment and then embedded in paraffin wax. After fixation in paraffin block, sections of 6µm thickness were prepared using microtome (Leica). Thus, sectioned specimens were stained using haematoxylin and eosin, after mounting on the glass slides. The slides were then microscopically examined for histological features.

Scanning Electron Microscopic Analysis

Experimental samples were cut from the official sampling position[17] and fixed in formalin solution. Then samples were dehydrated using ethanol and experimental samples were then gold coated using Edwards E 306 sputter coater and the specimens were examined using FEI Quanta 200 High-Resolution Scanning Electron Microscope. The orientations of the scanning electron micrograph for the cross section were obtained by operating microscope at high vacuum with an accelerating voltage of 12KV at different magnification levels.

Shrinkage Temperature of the Wet Blue Leathers

The experimental chrome tanned leathers were measured for shrinkage temperature according to the standard method.[18]

Physical and Organoleptic Properties of Leather

The experimental crust leathers were conditioned as per the standard procedure IUP 2.[17] The physical properties such as tear strength,[19] tensile strength and % elongation at break were measured. The bulk properties of all the experimental and control leathers, such as softness, fullness, grain flatness, grain smoothness and general appearance were evaluated by hand and visual examination. Each property of crust leathers was analyzed by three experienced technologists and results were expressed as rating on a scale of 0-10 points.

Results and Discussion

Qualitative Analysis of Bating

The alkaline protease from *Bacillus crolab* MTCC 5468 was assessed for its potential use in unhairing and avoiding bating step of leather processing (Experiment-I). Qualitative assessment of the air bubble test of the pelts, where the enzyme was used for unhairing step met the requirement of bating quality performance subsequently at the end of delimimg, and it was much better than conventionally unhaired skins without bating (Control-I); whereas enzymatically bated skins was comparable to that of bated skins even without bating.

In thumb impression test, on employing alkaline protease enzyme in the unhairing step (Experiment- I) it takes 24 sec., while the chemically unhaired skins (Control- I) take 18 sec. to reshape after imprint. In the case of Control-II and Experiment-II, on using commercial bate as a bating agent it takes 22 sec, while enzymatic bating takes 26 sec, respectively, to reshape after imprint on bated skins. The organoleptic properties of the conventionally unhaired skins without bating were unsatisfactory unlike other trials. Protease as a bating agent resulted in a highly silky, slippery feel and clean pelts when comparable to commercial bate treated skins.

Protein Content in Unhairing and Bating Spent Liquors

Alkaline protease used to remove dirt, scud and remove inter-fibrillary materials, so the relationship between enzymes and leather can be appraised by estimating the protein content of the bated liquor. Table I show that the protein content in the liquor of the enzymatically unhaired skins (Experiment-I) is higher than that in the conventionally unhaired skins (Control-I). Protein content of the Control-II is lower when compared to that of Experiment-II. The reason for the increased level of protein content in the liquor of the enzyme treated skins is due to the removal of non-leather forming materials by the alkaline protease compared to conventional materials.

Water Vapor Permeability

The values of water vapor permeability (WVP) of the crust leathers are shown in the Table II. Results indicates that the WVP of the enzymatically unhaired skins (Experiment-I) was higher than that of the conventionally unhaired skins (Control-I). Similarly, the WVP of the commercial bate treated skin (Control-II) was marginally lower than that of the enzymatically bated skins (Experiment-II). Higher WVP of the

Table I
Protein content in unhairing and bating spent liquors.

Experiments	Protein content (mg/kg of raw skins)
Unhairing experiment - I	2198.9 ± 51.7
Unhairing control - I	1570.5 ± 51.3
Bating experiment - II	2565.7 ± 47.5
Bating control - II	2055.8 ± 52.4

enzymatically bated leather is due to the reason that it results in a complete removal of scud and other inert-fibrillary materials leading to the free passage of water vapor through the collagen fibers, ensuring good acclimatization inside the footwear. In case of Control-I, the WVP of the crust leather was significantly low compared to other crust leathers as no enzyme treatment was provided.

Capillary Flow Porometry Analysis

Measurement of pore size and distribution can be determined rapidly by Capillary flow porometry (CFP) technique. The gas pressure is increased thus removing the liquid from the pore and allowing gas to flow through, which indicates the presence of throat pore.[20] The complete diffusion of leather chemicals into the skin matrix and comfort properties of leathers depend on the size of throat-pore.

In this regard, CFP measurements have been carried out for all the experimental goat wet blue leathers. The mean flow pore diameter values have been shown in the Table IV. It is evident from Figure 1, the enzymatically unhaired goat wet blue leather without bating have pore size in the range of 0.1-0.5 µm, whereas the conventionally unhaired skins without bating showed less pore size in the range 0.1-0.3µm. The commercial bate treated wet blue has pore size in the range of 0.1-0.4µm; whereas leathers where the new enzyme used as bate has wide range of pore size of 0.1-0.7 µm. The % distribution of pore sizes- 0.1µm and 0.2µm in the wet blue leathers of Experiment-I was found to be 27% and 22% and Control-I was found to be 29% and 10%; whereas Control-II showed 35% and 16% and Experiment-II showed 25% and 35% respectively. Based on the results it can be concluded that there is no significant difference between the wet blue leathers of Experiment-I and Experiment-II. These results indicate that the enzyme treated leathers has better porosity compared to other trials possibly due to the internal separation of collagen fibers by degradation of unwanted skin matrix substances.

Scanning Electron Micrograph Analysis

Grain surfaces and cross section of all experimental goatskins were observed at a magnification of 500x and images are given in Figure 4 respectively. The morphological analysis of enzymatically unhaired pelts (Experiment-I) were comparable to that of enzymatically bated skins (Experiment-II); while in the conventionally unhaired skins, the grain surface was rough and less opening of fiber structure. Both the commercial and enzyme bate treated skins exhibited smooth grain surface and better opened fiber matrix. From the SEM images, it can be concluded that the fiber bundles are loosened well and evenly spread in

Table II
Water vapour permeability.

Experiments	Water vapour permeability (mg/cm²hr)
Unhairing experiment - I	18.07 ± 1.5
Unhairing control - I	16.01 ± 1.2
Bating experiment - II	20.60 ± 1.9
Bating control - II	18.24 ± 1.3

Table III
Chrome content analysis and shrinkage temperature.

Experiments	Chrome content (% Cr_2O_3)	Shrinkage temperature (°C)
Unhairing experiment - I	4.19 ± 0.2	104 ± 1.0
Unhairing control - I	3.91 ± 0.1	102 ± 1.0
Bating experiment - II	4.27 ± 0.4	103 ± 1.0
Bating control - II	4.13 ± 0.1	105 ± 1.0

Table IV
Capillary flow porometry.

Experiments	Mean flow pore diameter (mm)
Unhairing experiment - I	0.175 ± 0.2
Unhairing control - I	0.118 ± 0.1
Bating experiment - II	0.199 ± 0.1
Bating control - II	0.131 ± 0.1

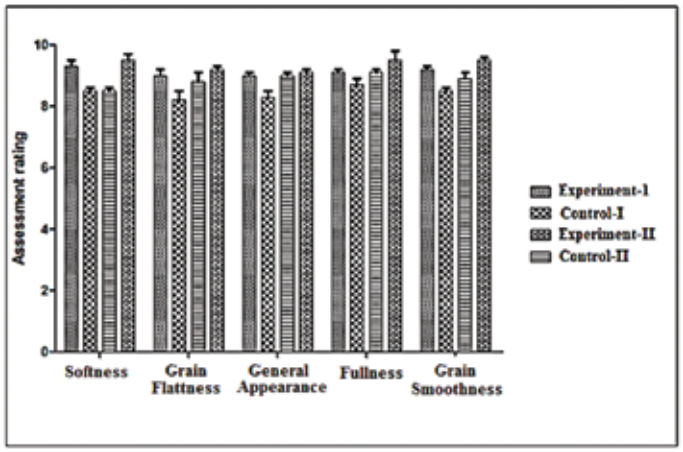

Figure 2. Visual assessment of experimental leathers.

enzyme treated pelts that confirmed the data obtained from histological evaluation (Figure 3), that the protease was able to penetrate efficiently into the skin and accomplish the removal of non-collagenous proteins and loosening of fiber structure. Paul*et al.*[21] reported that protease is well known for hydrolyzing the inter-fibrillary non-collagenous proteins and loosening of fiber network. So, this is considered as a benefit of enzymatic method over the conventional process.

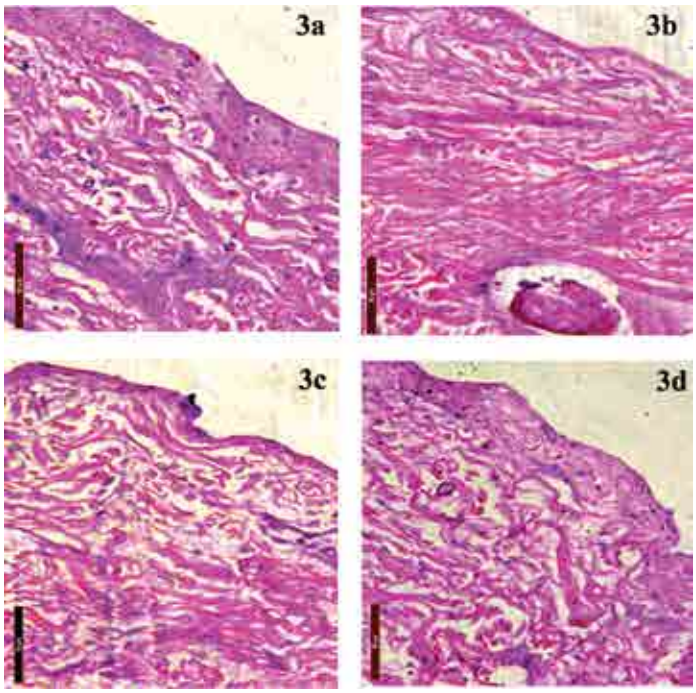

Figure 3. Histological analysis experimental pelt samples showing the cross section from Experiment-1 (3a), Control-I (3b), Experiment-II (3c), Control-II (3d).

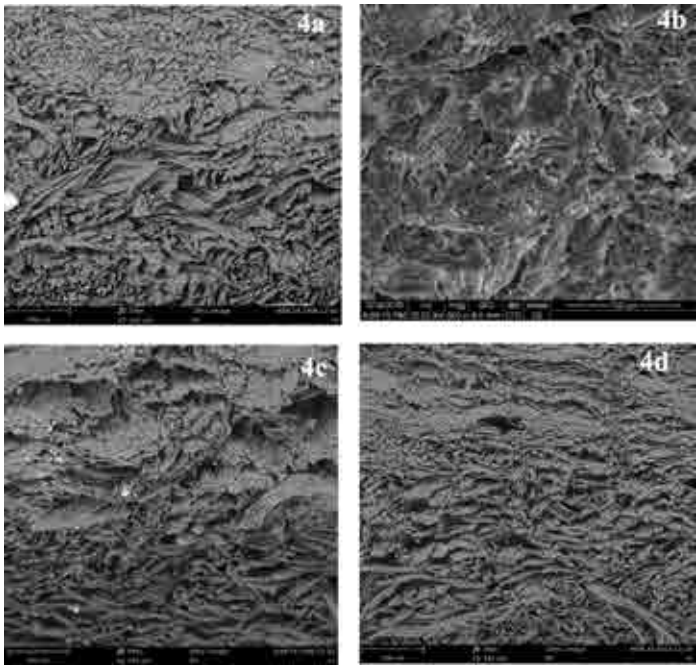

Figure 4. Scanning electron micrographs of pelt samples showing the cross section at a magnification of x500 from Experiment-1 (4a), Control-I (4b), Experiment-II (4c), Control-II (4d).

Histological Analysis

The bating efficacy of the crude alkaline protease was evaluated by histological analysis of the section of the dehydrated goat skins. The histology of all experimental pelts was shown in Figure 3. This reveals loosening of fiber matrix in the enzymatically unhaired pelt was better than conventionally unhaired pelt. While the fiber bundle loosening is equivalent to or better in the case of pelt bated with an enzyme than commercial bates treated pelt and enzymatically unhaired pelts. Whereas the section of chemically unhaired pelt implies the less opening of collagen fibers compared to that all experiments. This result indicates that alkaline protease alone can work as a very good bating agent in leather processing.

Physical and Organoleptic Properties of Leather

Samples were cut from the all the four experimental crust leathers and used for the measurement of physical properties such as tear strength, tensile strength, % of elongation at break and grain crack and shrinkage temperature (Table III). Table V shows the physical characteristics of crust leathers of all experiments. The properties of the enzymatically unhaired skins were much better than conventionally unhaired skins. The properties of enzyme treated (both I and II experiments) and commercial bate treated crust leather indicate that there was no significant difference between them. However, tensile strength, % elongation at break and shrinkage temperature of enzyme treated skins was considerably better when compared to commercial bate treated skins; this is because of the reason that enzyme would improve structural stability and mechanical strength of the leather. The physical characteristics of the conventionally unhaired skins were significantly low when compared to other experiments. According to the results of SEM and histological analysis, on using enzyme in bating process, a notable degree of opening of collagen bundles in the bated skins was observed.

The assessment of bulk properties of crust leathers showed that the rating for softness, grain flatness, smooth and fullness of enzymatically unhaired leather was superior to the conventionally unhaired leather; whereas the enzymatically bated unhaired leathers are equal to those of commercial bate treated leathers (Figure 2). The grain pattern and softness of enzyme treated skins are slightly better than the commercial bate treated skins. The organoleptic properties of conventionally unhaired leathers were not satisfactory unlike other experiments. So, using enzyme in bating and unhairing would improve the leather quality more when compared to that of other experimental leathers.

Table V
Physical properties of leather.

Experiments	Tensile strength (Kg/cm^2)	% elongation at break	Tear strength (Kg/cm)	Grain crack strength	
				Load (Kg)	Distension (mm)
Unhairing experiment - I	227.2 ± 0.2	43.8 ± 0.4	44.8 ± 0.8	33.9 ± 0.8	9.58 ± 0.8
Unhairing control - I	207.1 ± 0.9	41.1 ± 0.8	41.9 ± 0.2	31.6 ± 0.7	9.01 ± 0.4
Bating experiment - II	239.1 ± 0.1	45.1 ± 0.3	46.9 ± 0.9	35.9 ± 0.6	9.89 ± 0.5
Bating control - II	225.6 ± 0.7	42.4 ± 0.6	44.1 ± 1.0	33.7 ± 0.7	9.41 ± 0.5

Chrome Content

The chrome content of the wet blue leathers of all experiment has been estimated to evaluate chromium uptake capability of the pelts and it was listed in the Table III. Aforementioned results displays that the chrome uptake of the enzymatically unhaired skin was marginally better when compared to that of the conventionally unhaired skins; while enzymatically bated wet blue leathers were comparable to commercial bate treated leathers. This may probably be due to the loosened collagen bundles, better pore size, wherein more number of collagen cross linking sites were available for fixation with tanning. The chrome content in the conventionally unhaired skins was low compared to other experiments.

Conclusion

The alkaline protease of *Bacillus crolab* MTCC 5468 produced by solid state fermentation process was used for unhairing and bating operation. The advantage of enzymatic process includes complete removal of scud, opening up of collagen fiber matrix by removing the non- collagenous protein. This was confirmed by SEM, histological evaluation of the leather and protein content of the bated liquor. The extent of scud and non-collagenous protein removal of the enzyme treated skins was better than commercial bate treated leathers and conventionally unhaired skins. The physical and organoleptic properties of enzyme bated leather were comparable to those enzymatically unhaired leathers and better than that of commercially bated leathers. The study indicates when alkaline protease used for unhairing, bating operation can be avoided saving time and cost.

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