Textile & Leather Review

ISSN: 2623-6257 (Print) 2623-6281 (Online) Journal homepage: www.textile-leather.com Journal doi: 10.31881/TLR

Evaluation of the *Bacillus cereus* Strain 1-p Protease for the Unhairing of Goatskins during Leather Production

Joseph Ondari Nyakundi, Jackson Nyarongi Ombui, Francis Jakim Mulaa, Wycliffe Chisutia Wanyonyi

How to cite: Nyakundi JO, Ombui JN, Mulaa FJ, Wanyonyi WC. Evaluation of Bacillus cereus Strain 1-p Protease for the Unhairing of Goatskins during Leather Production. Textile & Leather Review. 2020. https://doi.org/10.31881/TLR.2020.18

How to link: https://doi.org/10.31881/TLR.2020.18

Published: 2 March 2021

Evaluation of the *Bacillus cereus* Strain 1-p Protease for the Unhairing of Goatskins during Leather Production

Joseph Ondari NYAKUNDI[1]*, Jackson Nyarongi OMBUI[1], Francis Jakim MULAA[2], Wycliffe Chisutia WANYONYI[3]

[1]Department of Public Health Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Nairobi, P.O. Box 30197 - 00100, Nairobi, Kenya
[2]Department of Biochemistry, College of Health Sciences, University of Nairobi, P.O. Box 30197 - 00100, Nairobi, Kenya
[3]Department of Physical Sciences, School of Science & Technology, University of Kabianga, P. O. Box 2030- 20200 Kericho, Kenyaa
*ondarijay@gmail.com

Article
UDC: 675.023.3:675.031.3:577.15
DOI: 10.31881/TLR.2020.18
Received 8 September 2020; Accepted 2 December 2020; Published Online 23 December 2020; Published 2 March 2021

ABSTRACT

The unhairing stage of leather processing is associated with the production of significant amounts of solid and liquid wastes. The use of enzymes to replace the polluting sulphides currently used for unhairing is a viable alternative. Various proteases from different Bacillus cereus strains as well as many other bacterial strains have been used successfully for the unhairing of skins. However, no previous work has assessed the use of the crude alkaline protease extract from Bacillus cereus strain 1-p, a novel Bacillus cereus strain obtained from the shores of Lake Bogoria - a soda lake in Kenya – in the unhairing of goatskins. This study, therefore, evaluates the potential of the protease extract from the Bacillus cereus strain 1-p to unhair goatskins.

Optimum variables for unhairing using the protease were investigated. Complete unhairing was achieved within 12 hours at 27°C and pH 12 using the crude enzyme. The period and temperature required for complete unhairing were significantly lower than that of other enzymatic unhairing techniques. Compared to the leather unhaired with sulphide, the leather unhaired with the enzyme did not only show superior organoleptic properties but also recorded comparable or superior physical properties, namely tensile strength (26.94 N/mm2), percentage elongation (76.29%), tear strength (43.59 N/mm), and distension at grain crack and burst (7.9 mm and 8.2 mm respectively). The wastewater from the enzymatic unhairing process recorded a significant reduction in biochemical oxygen demand (78%), chemical oxygen demand (83%), and the wastewater volume (50%) compared to the process that uses sulphide. It was concluded that the use of the crude protease extract from the Bacillus cereus strain 1-p in unhairing goatskins is feasible.

KEYWORDS

Bacillus cereus, Unhairing, Protease, Leather

INTRODUCTION

Leather is a valuable prehistoric natural commodity that is being used in modern times, and plays an indispensable economic role globally. Leather and finished leather products are estimated to have a global value of about USD 100 billion annually [1]. In the year 2013, footwear made of leather accounted for approxi-

mately USD 53.5 billion [2]. Despite making such substantial contributions towards economic development, the leather industry is blamed for severe environmental pollution resulting from the application of toxic agents in processing of hides and skins [3]. Leather processing entails the transformation of the biodegradable rawhide/skin into leather, a non-biodegradable product. This is through a series of processing stages that can be summarized into pre-tanning, tanning and post tanning [4,5].

The initial stages of leather processing generate 80–90% of wastes produced from tanneries [6]. Unhairing is a pre-tanning step in leather processing where toxic sulphide chemicals are used. This stage is thus associated with major environmental pollution. In the conventional unhairing technique, hides and skins are exposed to an extreme chemical treatment using lime and sodium sulphide that breaks down the hair and epidermal structures to remove them from the hide/skin [7]. As a result, this processing stage releases volumes of solid wastes in the form of broken epidermal structures and hair [8]. Additionally, this stage is responsible for air emissions that have negative effects on the environment, since some of the gases cause photochemical reactions, destruction of the ozone layer through the increase in greenhouse gases that lead to the formation of acid rain [9]. These concerns have necessitated the need for cleaner alternative technologies to eliminate the use of sulphides in the unhairing process.

Over the years, enzyme technology has made major strides in transforming several industrial processes into cleaner production systems. Enzymes have been used in food, textile, detergents and soaps, bioactive peptides, agricultural, cosmetic, beverage, pharmaceutical and leather industries, among others [10]. Microbial enzymes have been employed in tanneries for rehydration, hair removal, bating and removal of fats [7]. The application of enzymes for unhairing eliminates the use of the toxic sodium sulphide and can facilitate recovery of quality hair and fats which significantly lowers the pollution load in the effluent [10]. Enzymatic unhairing is associated with enormous benefits which include effective rehydration, scud loosening, splitting of collagen fibres, production of a smooth grain, improved soft feel making the leather pliable, improved yield, and overall reduction of the production time and pollution load [12-14].

Alkaline protease enzymes have widely been researched on, especially their application in leather processing. The use of proteases has played a vital role in various stages of leather manufacturing from soaking to the finishing stage [15]. Many Bacillus species have been explored for protease production with high potential strains distinguished to be *B. mojavensis*, *B. subtilis*, *B. licheniformis*, and *amyloliquefaciens* [16]. Research done on the enzymatic unhairing using enzyme extracts from the Bacillus species have reported satisfactory results on their efficacy in unhairing hides and skins [14,17-25].

Various researches have reported positive results on the use of proteases extracted from different *Bacillus cereus* strains for industrial applications such as unhairing hides and skins during leather processing. Great advances have been documented on the potential of *Bacillus cereus* BM1 protease in industrial applications, whereby it has proved to be thermostable and detergent compatible and, therefore, can find commercial applications [26]. Despite the protease's stability at an alkaline pH range of 8-12 which would be suitable for unhairing, the protease required a temperature range of 40-70°C for optimum activity, a temperature range that is too high for raw skins. A crude enzyme extract from *Bacillus cereus* VITSN04, whose maximum activity was reported to be at 30°C and pH 8.0, was found to be effective in unhairing of goat skins during leather processing [24]. Despite the remarkable results, this protease required 18 hours to achieve complete unhairing, a rather lengthy period in a typical production line. An extracellular protease extracted from a thermophilic *Bacillus cereus* strain isolated from soil demonstrated ability to completely remove glandular structures, hair and hair follicles from goatskins and cowhides at optimum conditions of pH 7.5 and temperature of 60°C [23]. The enzyme from this *Bacillus cereus* strain was not only active in the pH range of

6.0–9.0 but also at a relatively high-temperature range of 60-70°C, a temperature that would denature the raw collagen fibres in raw hides and skins during unhairing. In another study, an alkaline protease obtained from *Bacillus cereus* MCM B-326 was used to unhair a buffalo hide successfully at pH 7.0 and a temperature of 28 ± 2°C [27]. The protease, however, required 21 hours to achieve the complete unhairing of the hides. This period is quite lengthy for an ideal leather production line. A protease extracted from *Bacillus cereus* strain SZ-4 exhibited considerable hair removal capability on bovine, hircine, porcine and ovine skins within a period of 12 hours [28]. These were appreciable results as it was further reported that the enzyme did not change the structure of the dermis. The protease extract from the *Bacillus cereus* strain 1-p was reported to have successfully descaled a Nile perch skin (*Lates niloticus*) within a period of one hour and also reported positive results in the unhairing of a bull hide [25]. The results observed from the descaling process of Nile perch fish skins were remarkable as the descaling was achieved in a short time under a pH and temperature range that can be applied to raw skins without damaging the collagen structure.

Although extensive research has been carried out on enzymatic unhairing using extracts from various bacterial strains and more so, the *Bacillus cereus*, no work has been published on the enzymatic unhairing of goatskins by using a crude enzyme extract from a novel *Bacillus cereus* strain 1-p obtained from the shores of Lake Bogoria, a soda lake in the Rift Valley, Kenya. The working hypothesis is that the crude alkaline protease extracted from *Bacillus cereus* strain 1-p has the potential to unhair goatskins that would eliminate the use of sodium sulphide during leather processing. This is aimed at finding a viable alternative to the toxic chemicals used in the unhairing stage of leather processing, which would make leather-making cleaner and translate to a more sustainable industry.

MATERIALS AND METHODS

Materials

Wet salted goatskins were purchased from a local slaughterhouse in Nairobi, Kenya. Tanning and laboratory chemicals were acquired locally, while the isolated bacteria were cultured and the enzyme extracted at the Biochemistry Laboratory, University of Nairobi. The chemicals used in this study in the laboratory and tannery were of analytical and commercial grade. The crude protease was prepared from a *Bacillus cereus* bacteria, strain 1-p, which had previously been isolated, cultured and identified using the analysis of 16S r DNA sequencing method [25]. The crude protease from this bacterial strain was studied in this work following the positive results reported from its use in descaling of Nile perch skins [25].

Enzyme preparation

The enzyme was prepared at optimum conditions as described in a previous method [25]. A 3-litre medium was prepared with the following components: magnesium sulphate ($MgSO_4.7H_2O$), calcium chloride ($CaCl_2$), urea, yeast, casein, glucose and distilled water. The pH was adjusted to 11.5 and left to rest for two hours for pH stabilization before sterilization of the media and the flasks to be used. An overnight bacterium seed culture (*Bacillus cereus* strain *1-p*) in sterile distilled water was inoculated into the 3-litre medium setup in a bioreactor (Bioengineering RALF) set at 150 rpm, pH 11.5 and 45˚ C and incubated for 72 hours. The culture-medium was then obtained and centrifuged at 12,000 revolutions per minute for fifteen minutes to extract the supernatant. The supernatant was recovered and stored in covered flasks at 4°C awaiting use in the unhairing of goatskins.

Enzyme assay

The crude protease activity was investigated under optimized variables (pH 11 and 45°C) as described by Wanyonyi et al. (2015) following a modification of their method. The activity was determined using casein as the substrate at an alkaline pH. 0.1 ml of the crude enzyme was incubated with 0.1 ml of 1% w/v casein in the buffer-Tris-HCl (0.2 M, pH 9.0) at 45°C for 10 min. After the completion of the incubation, the reaction was terminated by addition of 4 ml of chilled trichloroacetic acid and placed on ice for 20 minutes to facilitate precipitation of insoluble proteins. The mixture was subsequently centrifuged for 10 minutes. 5 ml of 0.4 M sodium carbonate and 1 ml of diluted Folin-Ciocalteu reagent was added into the supernatant and incubated for 30 minutes. The absorbance was measured against a blank at 660 nm using a UV-spectrophotometer. One unit of the crude protease activity (U) was described as 1 µg of tyrosine liberated per minute per ml under standard assay conditions.

Optimization of unhairing

Factors affecting the unhairing of goatskins using the crude alkaline protease were studied and optimized to achieve maximum unhairing. The effect of temperature, pH and enzyme dilution was evaluated by dipping goatskin pieces in the crude enzyme and examining the extent of hair removal periodically. Three goatskins were cut into test pieces measuring 10x10 cm, which were washed and soaked in clean water for 2 hours in order to be rid of salt and dirt. The goatskin pieces were dipped in flasks with the 250 ml crude protease (0.45 U/ml) at 25°C and a pH range of pH 2 to 13 to assess the effect of pH on unhairing. A negative control experiment was also run in flasks under similar conditions but 250 ml of distilled water was used in place of the crude enzyme. The removed hair was weighed and expressed as a percentage against the total hair recovered after the complete unhairing. The percentage was established as per the area without hair compared to the test piece's total area. A complete hair removal was treated as 100% while no hair removal was 0%. The flasks, covered with cotton wool and aluminium foil, were shaken at intervals of 10 min. Gentle scrubbing using a blunt piece of wood was done to assess the ease of hair removal after every hour.

Goatskin pieces were dipped in flasks with 250 ml of crude protease (0.45 U/ml) at pH 12, within a temperature margin of 25°C - 35°C in order to study the effect of temperature on the unhairing. Similarly, a negative control experiment with 250 ml of distilled water in place of the crude enzyme at pH 12 was run within the same temperature range. The flasks, covered with cotton wool and aluminium foil, were shaken at intervals of 10 minutes and the ease of hair removal was determined after every hour.

Other goatskin test pieces were dipped in flasks with the crude protease diluted in different proportions; 100% (250 ml of crude enzyme – 0.45 U/ml), 80% (200 ml of crude enzyme – 0.45 U/ml, diluted with 50 ml of distilled water), 60% (150 ml of crude enzyme – 0.45 U/ml, diluted with 100 ml of distilled water), 40% (100 ml crude enzyme – 0.45 U/ml, diluted with 150 ml of distilled water), 20% (50 ml of crude enzyme – 0.45 U/ml, diluted with 200 ml of distilled water) and 0% (250 ml of distilled water), all adjusted to pH 12 to investigate the impact of enzyme dilution on unhairing. The flasks were observed at 27°C to allow the enzyme activity to take place. The ease of hair removal was determined at intervals of one hour.

Enzymatic leather processing

Once the unhairing conditions had been optimized, the unhairing of full goat skins was done with the crude alkaline protease at the optimized conditions at a local tannery. Experimental tannery drums were used to process a total of nine (9) goatskins in replicates of three using the enzyme extract. A positive control

experiment was also set up to unhair 9 goatskins using sodium sulphide, also in triplicates. All the skins were washed thoroughly and soaked for 16 hours in the presence of a 0.2% biocide, 0.1% wetting agent and 0.5% soda ash before the start of the unhairing process. The amount of all the chemicals used was calculated as a percentage of raw weight. A complete unhairing in the experimental group was achieved using the undiluted crude protease, at the optimized pH 12 and 27°C, in 12 hours. The pelt was gently scraped using a blunt blade to remove the hair, which was recovered, washed and dried. The pelts were then limed and subsequently fleshed for the recovery of sulphide-free flesh and fats. Similarly, the positive control group was conventionally unhaired using the lime-sulphide system and fleshed. The subsequent pre-tanning, tanning and post-tanning stages were carried out in similar standard treatments for the sulphide- unhaired and the enzyme-unhaired goatskins. Notably, the experimental group was not subjected to the bating process after deliming as the pelts were clean and well opened up, unlike the control group. The chrome-tanned leathers were processed to crust leather as per a standard procedure.

Organoleptic examination and physical tests of the processed leather

The enzyme-unhaired leathers were examined for visual and physical properties and compared to the lime–sulphide unhaired leathers. Various organoleptic properties that include appearance, softness, fullness, wrinkles, colour intensity and uniformity of the enzyme- unhaired and the sulphide-unhaired leathers were examined visually and through touch. They were then rated from 0 to 10 points for every examined parameter by three experienced leather technologists with higher points representing a superior quality. Leather samples for physical testing were acquired through a standard sampling procedure as stipulated by the ISO 2418:2002 standard [29]. Eighteen (18) pieces of the enzyme-unhaired goatskin leathers were cut by applying the standard dumbbell-shaped press knife to the grain surface. Nine (9) test pieces were obtained with the longer side parallel to the backline and nine (9) with the longer edge perpendicular to the backbone. These test samples were conditioned in a standard atmosphere and standard conditions; at the temperature of 23 ± 2°C and the humidity of 50 ± 5% for 48 hours as specified by the ISO 2419:2002 standard before testing [30]. The physical tests, such as tensile strength, tear strength and grain distension were tested as per the International Union for Physical testing for leather (IUP) methods. Tensile strength and elongation were tested in line with the ISO 3376:2002 standard [31]. The jaws of the tensile testing instrument were set apart and the samples secured in-between, aligning the jaw clamps with the midline. The machine was run and the jaws began to separate, stretching the test piece until it broke and the load recorded. The tear strength of the leather samples was measured by the Instron testing machine following the standard test method ISO3377-2:2002 [32]. A notched sample was clumped between the machine jaws. Upon the running of the machine, the jaws separated until the test piece tore and the load recorded. The strength of the grain was tested as per the standard ISO 3379:2015 [33]. A disk-shaped test piece was distended by pushing through the flesh side and observing the grain surface for incipient cracking and bursting and subsequently recorded as the distension at grain crack and distension at grain burst respectively.

Analysis of resultant liquor

The resultant wastewaters from the control (sulphide unhairing) and the experimental (enzymatic unhairing) groups were obtained after the unhairing stage and analyzed for biochemical oxygen demand (BOD), chemical oxygen demand (COD), total dissolved solids (TDS) and total suspended solids (TSS) following the standard technique [34].

Data analysis

The data were analyzed by using the IBM SPSS Statistics 20 statistical tool. The T-test analysis was done to compare the means of the physical properties of the enzyme-unhaired goatskin leathers to the conventionally processed ones to assess the statistical difference of the results obtained (Significance level; $\alpha < 0.05$). The differences were concluded to be significant when $p<0.05$.

RESULTS AND DISCUSSION

Effect of the pH on enzymatic unhairing

The crude enzyme extracted after the 72h fermentation was assayed for its proteolytic activity. It was found that the activity of the crude protease from *Bacillus cereus* strain *1-p* was 0.45 U/ml at the optimized variables. Hydrogen ion concentration (pH) is well known to affect the enzyme activity, because enzymes are proteins in nature, thus sensitive to changes in the pH [35]. The effect of the pH on the enzymatic unhairing of goatskins was studied at the pH range 2-13 at 25°C and the observations were summarized in Figure 1. The results showed that at a low pH (acidic), there was no hair removal throughout the observation period. Partial hair removal was first recorded at pH 12, 11, 9, 8 and pH 7 after 2, 4, 5, 5 and 6 hours respectively. Complete hair removal was only observed at pH 12 and 11 after 7 and 12 hours respectively. Negative control experiments containing distilled water in place of the crude enzyme at a pH range of 2 to 12 showed no removal, indicating that the crude protease enzyme was solely responsible for the unhairing. The observed increase in unhairing efficiency with the increase in pH can be attributed to the increased effect on ionic and hydrogen bonds of the protease, which are important to the enzyme shape and its activity [36]. The observed results correspond to the properties of alkaline enzymes previously reported to show the highest unhairing rate at pH 8 to pH 12 [23-25,37-40].

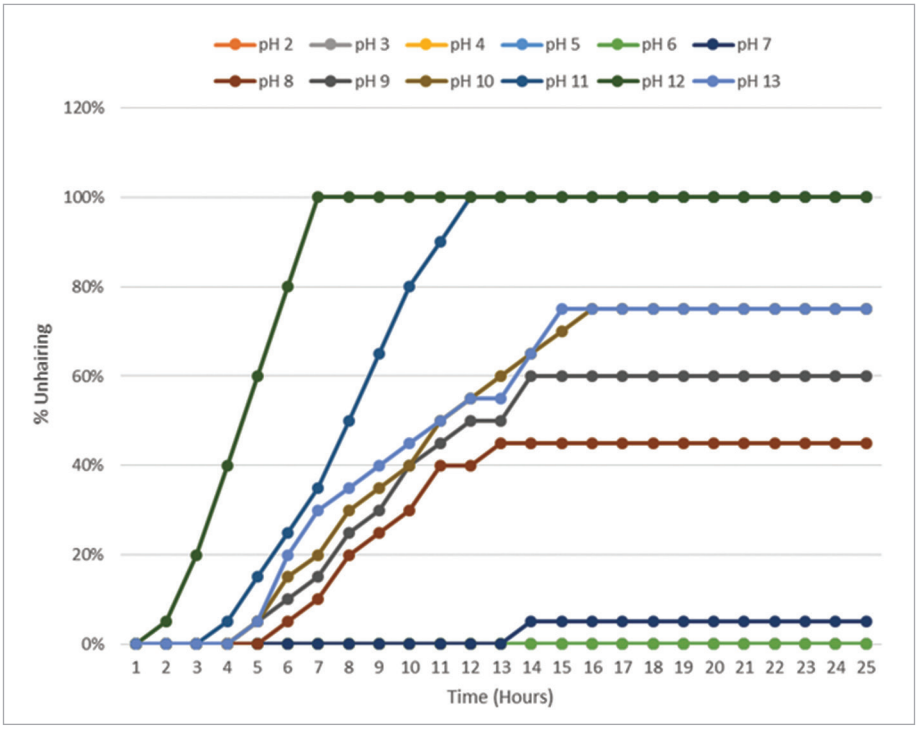

Figure 1. Effect of pH on unhairing by using the enzyme from ***Bacillus cereus*** strain ***1-p***

Effect of temperature on the enzymatic unhairing

Increase in temperature increases the reaction rate up to a specific temperature limit, beyond which the enzyme is denatured [41]. The effect of temperature on enzymatic unhairing of goatskins was investigated within the range of 25°C–35°C, at pH 12 using the undiluted crude protease, and the results displayed in Figure 2. The unhairing efficiency increased with an increase in the temperature. 100% hair removal was attained after 5 and 6 hours at 35°C and 33°C respectively. At 27°C and 25°C, complete unhairing was achieved after 7 hours. The negative control experiments showed no hair removal. These findings are significantly positive as hair removal was achieved in an acceptable temperature range for the raw hides and skins in a significantly short period. High temperatures are avoided in the early stages of leather processing because the shrinkage/denaturation temperature for raw skins is approximately 65°C around neutral pH while at high pH the skin's shrinkage temperature reduces to 50°C or less; it is thus recommended that the temperature for the soaking, unhairing and liming of skins must be limited to less than 30°C [42]. These observations showed a significant potential of the protease enzyme from the *Bacillus cereus* strain *1-p* to be used for unhairing. These results are better than those reported of other microbial enzymatic unhairing techniques that required significantly higher temperature ranges to achieve complete hair removal [14,17,23].

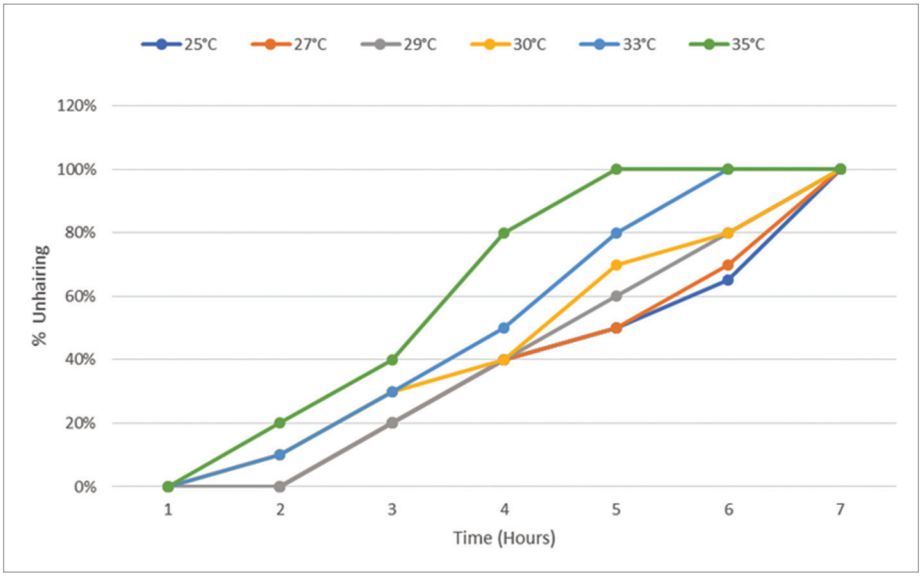

Figure 2. Effect of temperature on unhairing by using the enzyme from *Bacillus cereus* strain *1-p*

Effect of the enzyme activity on the unhairing activity

Enzyme concentration affects the rate of enzyme activity by altering the number of enzyme molecules present. The effect of the enzyme dilution on unhairing of goatskins was determined at 27°C and pH 12 using proportions of the crude enzyme diluted using distilled water and the results presented in Figure 3. The results show that the protease unhairing efficiency decreased with the increase in the crude enzyme dilution. Complete hair removal was first observed in the ratio 100:0 after 7 hours while the ratios 80:20 and 60:40 took 9 hours and 10 hours respectively to achieve complete unhairing. This can be ascribed to the fact that the enzyme activity is highly dependent on its molecular concentration, which means that a high enzyme concentration translates to high enzyme activity [43]. Although various factors affecting the enzymatic unhairing have been investigated and are well established, the effect of the enzyme dilution has not been extensively studied and documented. Nonetheless, as an increase in dilution generally lowers

the concentration of enzyme molecules present in a specific volume, these findings correspond to those reported from the use of a protease extract from *Bacillus crolab MTCC 5468* to unhair goatskins where the unhairing activity and efficiency increased with the increase in enzyme concentration, thus a complete unhairing was achieved faster [7].

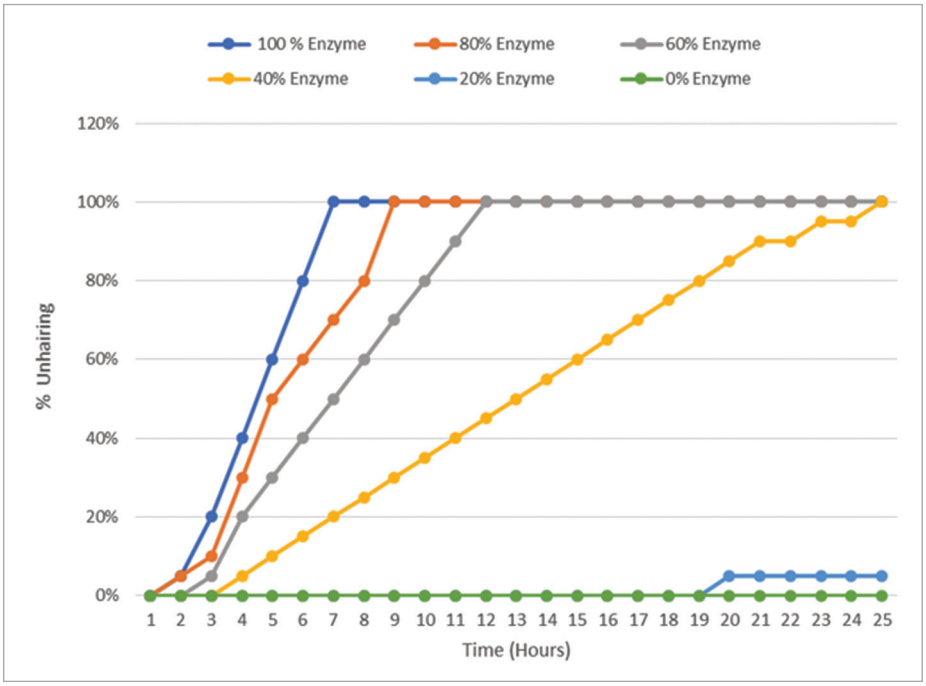

Figure 3. Effect of the enzyme activity on the unhairing by using the enzyme from ***Bacillus cereus*** strain ***1-p***

Unhairing of full goatskins

The established optimum variables were used to unhair full goatskins by using the crude protease derived from *Bacillus cereus* strain *1-p* at a local tannery. A positive control run was set up, where goatskins were unhaired using sodium sulphide. It was noted that the crude protease achieved complete unhairing in 12 hours without the emission of any pungent smell. This was slightly longer than the period (7 hours) required to completely unhair test pieces at the laboratory scale. This can be attributed to the varying processing conditions, such as the temperature and the pH in the tannery processing drum. The novel protease from *Bacillus cereus* strain *1-p* recorded a relatively shorter unhairing period compared to the ones reported from previous studies on alkaline proteases that reported unhairing periods of 16 hours up to 24 hours [13,14,22,44,45].

Visual examination of the pelts after the completion of the unhairing process showed that the pelt from the experimental group (enzyme-unhaired) was whiter and had a cleaner grain surface, void of hair follicles and epidermal structures, compared to the control group (sulphide-unhaired), which had darker patches, as displayed in Figure 4. This necessitated a further process step, bating of sulphide-unhaired pelts, the aim of which was to make the grain surface cleaner by removing scud and epidermal debris [46]. These results are in agreement with those reported by the previous research studies [8,13,47,48].

(a) (b)

Figure 4. Comparison between the enzyme-unhaired pelt (a) and the sulphide-unhaired pelt (b)

A significant amount of intact hair was recovered from the enzymatic unhairing system while the sulphide system produced hair that was turned into a sludge-like mass (pulped form) with no distinctive structure. The hair was easily sieved out of the unhairing liquor, thus reducing the solid waste in the resultant effluent stream. Similar observations have been reported from previous enzymatic unhairing studies, whereby undamaged hair was recovered, which translated to the overall reduction of the pollution load in the waste-water [13,48-50].

Both groups of unhaired pelts were further processed, tanned into wet-blue and post-tanned into crust leather as displayed in Figure 5. The physical properties of the experimental and control groups were tested and compared.

a) Raw wet-salted goatskins b) Wet-blue goatskin leather c) Crust goatskin leather

Figure 5. Enzymatically processed goatskin from the raw stage (a), through wet-blue (b) to crust stage (c)

Organoleptic and physical properties of the resultant leather

Physical properties of leather are vital quality parameters that determine the leather's performance characteristics in various applications, while organoleptic features indicate the general appeal of leather. The overall properties of the enzyme-processed leathers were either similar or better compared to the sulphide-processed leathers. Organoleptic properties of leather make the product unique and valuable for various applications.

Organoleptic properties

The visual and hand assessment of the conventional and the enzyme-unhaired leathers by experienced leather technologists showed that the enzyme-unhaired leathers were comparable or marginally better in appearance, softness, fullness, colour intensity and uniformity as presented in Table 1. The enzyme-unhaired leathers generally showed superior properties in the assessed parameters with the following ratings: appearance (9.5), softness (9.5), fullness (9.2) and colour intensity and uniformity (9.6) compared to the conventionally processed leathers with the ratings: appearance (8.9), softness (8.5), fullness (8.8) and colour intensity and uniformity (9.2). It was also notable that wrinkles were more prevalent in the enzyme-unhaired group (rated 9.8) compared to the sulphide-unhaired group (rated 9.0).

Table 1. Visual and hand assessment of the conventional and the enzyme-unhaired leathers

	Appearance		Softness		Fullness		Wrinkles		Colour Intensity & Uniformity	
	Conv.	Enzy.	Conv.	Enzy.	Conv.	Enzy.	Conv.	Enzy.	Conv.	Enzy.
Rating (out of 10)	8.9	9.5	8.5	9.5	8.8	9.2	9.0	9.8	9.2	9.6

Where: Conv. – Conventional (sulphide) unhairing; Enzy. – Enzymatic unhairing

The enzyme-unhaired leather was of good quality characterized by uniform colour, smooth grain, appreciable flexibility and a good appearance without any scud. This general observation has been attributed to the enhanced uptake of processing chemicals following the sufficient opening up of the fibre matrix as documented by previous research on enzymatic unhairing that reported similar results [7,12,13,18,47].

The general appearance of the conventionally and enzymatically treated goatskin leathers had a clear and clean grain without any foreign materials, but the enzyme-unhaired leathers were rated higher for the smooth feel, the gloss of the grain surface, a good handle, colour evenness and the general fullness of the leather structure. This can also be attributed to the enhanced absorption of tanning chemicals following the adequate splitting of collagen fibres. Similar observations on the appearance and fullness of the enzyme-unhaired leather have previously been reported [7]. The 'fibres splitting' effect of the enzyme processing also influences the uptake of colour and dye material, thus giving the leather the observed deeper and more uniform colour [14].

The average ratings on the softness of the leathers suggest that the enzymatically processed leathers were much softer than the sulphide-processed leathers. Enzymatically processed leathers have previously been reported to be softer due to the enzyme's action on non-structural proteins [12, 51]. This implies that enzyme application softens leather and makes it more pliable to the feel and touch. Leather softness can be associated with the fibre opening up [52]. Besides fully opening up the collagen structure, it is reported that enzymatic unhairing degrades the elastin network as opposed to the lime-sulphide system [48]. The

soft feel of the leather produced by the enzymatic unhairing process, therefore, can be explained by the removal of elastin, making it more flexible, supple and pliable. This is a desirable feature for the production of soft and pliable products like upholstery, garment and glove leathers. Similar observations on the enzyme-unhaired leathers being softer compared to the sulphide-unhaired ones have been reported [53]. The enzyme-unhaired leathers were also characterized by many wrinkles compared to the sulphide-unhaired leathers. The degradation of the elastin can also explain the evident prevalence of wrinkles in the enzyme-processed leathers. Elastin is an important protein that enables the skin to resume its shape after stretching or contracting. Hence, after its degradation, the leather surface does not regain its shape after distortion. Similar observations were made when an enzyme extract from *Bacillus crolab MTCC 5468* was used to process goatskins [7].

Physical properties

Table 2 shows the physical properties of the resultant crust leathers. The general outlook of the sulphide-unhaired and the enzyme-unhaired leather was comparable. The physical properties of both groups of leather met the minimum recommended standards [54]. The leathers obtained from enzymatic unhairing showed comparable tensile strength, distension at grain crack and distension at grain burst values to sulphide-unhaired leathers with no significant difference between them (p values 0.81, 0.489 and 0.147 respectively). The elongation at break and tear strength values for the experimental leathers showed that there may be a significant difference when compared to the control samples (p values 0.026 and 0.037 respectively).

Table 2. Physical properties of the leathers from the enzymatically and the sulphide-unhaired goatskins

	Tensile Strength (N/mm^2)			Elongation (%)			Tear Strength (N/mm)			Grain Distension (mm)	Grain Distension (mm)
	↑	→	Mean	↑	→	Mean	↑	→	Mean	Crack	Burst
Conventional unhairing	33.01	16.87	24.94	64.79	70.46	67.63	40.16	42.70	41.43	7.79	8.01
Enzymatic unhairing	33.73	20.13	26.93	65.68	86.89	76.29	42.37	44.81	43.59	7.89	8.23

Where: → - Perpendicular to the backline (transverse); ↑ - Parallel to the backline (longitudinal)

The comparability of the physical properties can be attributed to the fact that both unhairing techniques preserve the collagen content without causing damage. These results show that the crude protease does not compromise the strength of the collagen structure and therefore the technique is effective and practically feasible. Additionally, these results correspond to the properties of enzymatically unhaired leathers previously described, showing comparable or superior physical properties when compared to sulphide-unhaired leathers [13,47,48]. Notably, the grain distension resistance of the enzyme-unhaired leathers was better than that of the sulphide-unhaired leathers as presented in Table 2. This can be attributed to the fact that the enzyme action is less harsh to the grain layer compared to the sulphide action and also the enhanced absorption of tannins, which has been previously observed and reported in the enzymatically processed leathers [7,18].

Further observations were made concerning the sampling direction of the samples obtained for physical testing. Similar trends were observed in both the enzyme-unhaired and the sulphide-unhaired leathers concerning the sampling direction for tensile strength, percentage elongation and tear strength, as shown

by the results in Table 2. The tests pieces sampled parallel to the backbone recorded higher tensile strength values (sulphide-unhaired: 33.01 N/mm^2 and enzyme-unhaired: 33.73 N/mm^2) than the perpendicularly-sampled test pieces (sulphide-unhaired: 16.87 N/mm^2 and enzyme-unhaired: 20.13 N/mm^2). For the samples cut parallel to the backbone, most of their fibres are aligned in the same direction as the applied stress, hence having very little room to orientate towards the strain axis [55]. This means that the leather fibres themselves are strained, as there is minimal strain/stretch gained, as there is a minimal orientation of the fibres, explaining the higher tensile strength values. These conclusions are in agreement with those observed on the enzyme-unhaired leathers and the general behaviour of leathers during physical testing with regard to the sampling direction [13,56].

On the contrary, the samples cut perpendicularly to the backbone had higher percentage elongation and tear strength than those sampled parallel, as shown in the results in Table 2. The percentage elongation values (sulphide-unhaired: 64.79% and enzyme-unhaired: 65.68%) and the tear strength values (sulphide-unhaired: 40.16 N/mm and enzyme-unhaired: 42.37 N/mm) of test pieces cut parallel to the backline were lower than the percentage elongation values (sulphide-unhaired: 70.46% and enzyme-unhaired: 86.89%) and tear strength values (sulphide-unhaired: 42.70 N/mm and enzyme-unhaired: 44.81 N/mm) of samples cut perpendicular to the backline. For the test pieces cut parallel to the backline, the fibre network is assumed to be naturally orientated to the same direction as the strain axis. In this case, the specific work of fracture was higher because the tear does not grow through the fibre diameters but instead propagates in a more straightforward rupturing process and hence the tear strength is low [56]. The percentage elongation is equally lower in these samples as the stress acts on the leather fibres themselves, which are strained with minimal stress. Similar findings concerning sampling direction when carrying out physical tests have been reported [48,55-57].

Pollution load in resultant wastewater

Wastewaters from both unhairing techniques were assessed and the results are presented in Table 3. The results are expressed in parts per million (ppm).

Table 3. Pollution load in unhairing wastewater streams

Pollution loads (ppm)	Enzymatic unhairing group	Sulphide unhairing group	Percentage reduction of pollution (%)
BOD	940	4 200	78
COD	3 200	19 200	83
TDS	12 800	50 400	75
TSS	1 300	3 460	62
Total wastewater volume from the unhairing process	44 litres	88 litres	50

The presence of organic matter in wastewater contributes to increased BOD, COD, TDS and TSS [7]. It is clear from the results that the enzymatic unhairing, using the crude protease from *Bacillus cereus* strain *1-p,* leads to a significant reduction in BOD by 78%, COD by 83%, TDS by 75% and TSS by 62%. It was equally notable that the volume of the wastewater produced after the unhairing process was reduced by 50% in the enzymatic technique. The hair shafts were removed completely intact and recovered from the unhairing liquor; hence the wastewater was free from hair and epidermal matter unlike in the conventional technique. The significant reduction in the pollution load can be attributed to the complete elimination of sodium sulphide

which solubilizes the hair and epidermis during the unhairing. These findings agree with previous findings from enzymatic unhairing systems that reported that enzymes drastically shrink the effluent pollution load and help in hair recovery [25]. Similar results have been reported from proteolytic alkaline unhairing systems indicating a significant reduction of the pollution loads from the unhairing wastewater streams [7,14]. However, the pollution load reduction from the unhairing process using this novel protease recorded a higher reduction compared to that reported by Senthilvelan et al. (2012), with a 62.8% and 79.0% reduction in BOD and COD respectively. Hence, the protease extract from *Bacillus cereus* strain *1-p* is a viable and potential alternative for cleaner leather production.

The overall cost of leather production to the crust stage after the unhairing using the crude protease was approximately 0.55 USD per square foot compared to the cost of the conventional processing which was approximately 0.49 USD per square foot. The cost of the enzymatic unhairing technique was estimated from an inclusive calculation of the costs of all the laboratory reagents used for the enzyme extraction together with the processing chemicals at the tannery. Despite the slightly higher cost of production, the enzyme technique significantly reduced the pollution load, which would reduce the cost of effluent treatment and thus, give it an edge over the conventional technique.

CONCLUSION

This *Bacillus cereus* strain demonstrated the ability to secrete proteases that acted on the epidermal structure and hair, thus enabling their removal. The complete unhairing of the goatskins using the crude protease extract from *Bacillus cereus* strain 1-p took 12 hours at a relatively low temperature of 27°C. This unhairing technique yields leather that has comparable or even superior strength properties as well as organoleptic properties such as softness, fullness and colour uniformity when compared to the sulphide unhairing technique. The enzyme also facilitated the removal of the hair intact, which not only lowers the pollution load in the wastewater but also leads to recovery of the hair, which can be used for other industrial applications. The novel crude alkaline protease enzyme from *Bacillus cereus* strain 1-p can, therefore, be used to make leather processing cleaner and sustainable by eliminating the use of sodium sulphide without compromising on the quality of the resultant leather, and should, therefore, be explored for industrial applications and commercialization.

Acknowledgments

The authors wish to thank Bio-Innovate Africa for their financial support of this study. The authors acknowledge the Leather Industries of Kenya for their kind collaboration in the processing of samples for the study. Special thanks to the University of Nairobi staff and colleagues from the Department of Public Health Pharmacology and Toxicology and the Department of Biochemistry.

REFERENCES

[1] UNIDO. United Nations Industrial Development Organization, Vienna: Future Trends in the World Leather and Leather Products Industry and Trade [Internet]. 2010 [cited 2019 Nov 4]. Available from:https://leatherpanel.org/sites/default/files/publications-attachments/future_trends_in_the_world_leather_and_leather_products_industry_and_trade.pdf

[2] Hansen ER, Moon Y, Mogollon MP. Kenya - Leather industry : diagnosis, strategy, and action plan [Internet]. The World Bank; 2015 Jun [cited 2019 Nov 4] p. 1–126. Report No.: 99485. Available from:http://documents.worldbank.org/curated/en/397331468001167011/Kenya-Leather-industry-diagnosis-strategy-and-action-plan

[3] George N, Chauhan PS, Kumar V, Puri N, Gupta N. Approach to ecofriendly leather: characterization and application of an alkaline protease for chemical free dehairing of skins and hides at pilot scale. Journal of Cleaner Production. 2014 Sep;79:249–57. https://doi.org/10.1016/j.jclepro.2014.05.046

[4] Dixit S, Yadav A, Dwivedi PD, Das M. Toxic hazards of leather industry and technologies to combat threat: a review. Journal of Cleaner Production [Internet]. 2015 Jan [cited 2019 Oct 9];87:39–49. https://doi.org/10.1016/j.jclepro.2014.10.017

[5] Musa AE, Gasmelseed GA. Development of eco-friendly combination tanning system for the manufacture of upper leathers. International Journal of Advance Industrial Engineering. 2013;1(1):9–15.

[6] Thanikaivelan P, Rao JR, Nair BU, Ramasami T. Progress and recent trends in biotechnological methods for leather processing. Trends in Biotechnology. 2004 Apr;22(4):181–8. https://doi.org/10.1016/j.tibtech.2004.02.008

[7] Ranjithkumar A, Durga J, Ramesh R, Rose C, Muralidharan C. Cleaner processing: a sulphide—free approach for depilation of skins. Environmental Science and Pollution Research. 2017;24(1):180–188. https://doi.org/10.1007/s11356-016-7645-6

[8] Tian J, Xu Z, Long X, Tian Y, Shi B. High-expression keratinase by Bacillus subtilis SCK6 for enzymatic dehairing of goatskins. International Journal of Biological Macromolecules. 2019 Aug;135:119–26. https://doi.org/10.1016/j.ijbiomac.2019.05.131

[9] Hashem A, Arefin S, Jor A. Gaseous Air Pollutants and its Environmental Effect- Emittedfrom the Tanning Industry at Hazaribagh, Bangladesh. American Journal of Engineering Research. 2015;7.

[10] Contesini FJ, Melo RR de, Sato HH. An overview of Bacillus proteases: from production to application. Critical Reviews in Biotechnology. 2018 Apr 3;38(3):321–34. https://doi.org/10.1080/07388551.2017.1354354

[11] Arunachalam C, Saritha K. Protease Enzyme: an Eco-friendly Alternative for Leather Industry. Indian Journal of Science and Technology [Internet]. 2009 Dec 1 [cited 2019 Oct 28];2(12):29-32–32. Available from: http://www.indjst.org/index.php/indjst/article/view/29553

[12] Kandasamy N, Velmurugan P, Sundarvel A, Jonnalagadda Raghava R, Bangaru C, Palanisamy T. Eco-benign enzymatic dehairing of goatskins utilizing a protease from a Pseudomonas fluorescens species isolated from fish visceral waste. Journal of Cleaner Production [Internet]. 2012 Apr [cited 2020 Apr 22]; 25:27–33. https://doi.org/10.1016/j.jclepro.2011.12.007

[13] Khandelwal HB, More SV, Kalal KM, Laxman RS. Eco-friendly enzymatic dehairing of skins and hides by C. brefeldianus protease. Clean Technologies and Environmental Policy. 2015;17(2):393–405. https://doi.org/10.1007/s10098-014-0791-y

[14] Senthilvelan T, Kanagaraj J, Mandal AB. Application of enzymes for dehairing of skins: cleaner leather processing. Clean Techn Environ Policy [Internet]. 2012 Oct 1 [cited 2019 Oct 10];14(5):889–97. https://doi.org/10.1007/s10098-012-0458-5

[15] Mukhtar H, Haq I. Optimization of volume of fermentation medium for the production of alkaline protease by an ems mutant strain of bacillus subtilis ih72. Pakistan Journal of Botany. 2007; 39:2705–15.

[16] Mienda BS, Yahya A, Galadima IA, Shamsir MS. An overview of microbial proteases for industrial applications. Research Journal of Pharmaceutical, Biological and Chemical Sciences. 2014;5:388–396.

[17] Haddar A, Hmidet N, Ghorbel-Bellaaj O, Fakhfakh-Zouari N, Sellami-Kamoun A, Nasri M. Alkaline proteases produced by Bacillus licheniformis RP1 grown on shrimp wastes: application in chitin extraction, chicken feather-degradation and as a dehairing agent. Biotechnology and Bioprocess Engineering. 2011;16(4):669. https://doi.org/10.1007/s12257-010-0410-7

[18] Liu J, Holmes G. Biotechnology for environmently-friendly leather production. XXXV. Congress of IULTCS; June 25th-28th Dresden, Germany. 2019.

[19] Nadeem M, Qazi JI, Baig S. Enhanced production of alkaline protease by a mutant of Bacillus licheniformis N-2 for dehairing. Brazilian Archives of Biology and Technology. 2010;53(5):1015–1025. http://dx.doi.org/10.1590/S1516-89132010000500003

[20] Pillai P, Archana G. Hide depilation and feather disintegration studies with keratinolytic serine protease from a novel Bacillus subtilis isolate. Applied Microbiology and Biotechnology. 2008;78(4):643. https://doi.org/10.1007/s00253-008-1355-z

[21] Prakash P, Jayalakshmi SK, Sreeramulu K. Production of keratinase by free and immobilized cells of Bacillus halodurans strain PPKS-2: partial characterization and its application in feather degradation and dehairing of the goat skin. Applied biochemistry and biotechnology. 2010;160(7):1909–1920. https://doi.org/10.1007/s12010-009-8702-0

[22] Rajkumar R, Jayappriyan KR, Rengasamy R. Purification and characterization of a protease produced by Bacillus megaterium RRM2: application in detergent and dehairing industries. Journal of basic microbiology. 2011;51(6):614–624. https://doi.org/10.1002/jobm.201000517

[23] Saleem M, Rehman A, Yasmin R, Munir B. Biochemical analysis and investigation on the prospective applications of alkaline protease from a *Bacillus cereus* strain. Molecular biology reports. 2012;39(6):6399–6408. https://doi.org/10.1007/s11033-011-1033-6

[24] Sundararajan S, Kannan CN, Chittibabu S. Alkaline protease from *Bacillus cereus* VITSN04: Potential application as a dehairing agent. Journal of bioscience and bioengineering. 2011;111(2):128–133. https://doi.org/10.1016/j.jbiosc.2010.09.009

[25] Wanyonyi WC, Shiundu, Paul M., Onyari, John Mmari, Mulaa, Francis J. Isolation and characterization of biomass modifying enzymes for bioremediation and production of "green specialty products." 2015.

[26] Mienda BS, Huyop F. Characterization of *Bacillus cereus* BM1 with protease activity. Research in Biotechnology. 2013;4(3). Available from: http://updatepublishing.com/journal/index.php/rib/article/view/2430.

[27] Zambare VP, Nilegaonkar SS, Kanekar PP. Production of an alkaline protease by *Bacillus cereus* MCM B-326 and its application as a dehairing agent. World Journal of Microbiology and Biotechnology [Internet]. 2007 Nov 1;23(11):1569–74. https://doi.org/10.1007/s11274-007-9402-y

[28] Yang S, Zhou R. A Potential Unhairing Protease from a Newly Isolated Bacillus cereus. Agricultural Biotechnology. 2012;1(6):22.

[29] IULTCS/IUP 2. ISO 2418:2002 [IULTCS/IUP 2] Leather — Chemical, physical and mechanical and fastness tests — Sampling location [Internet]. ISO. 2002 [cited 2020 Feb 24]. Available from: https://www.iso.org/cms/render/live/en/sites/isoorg/contents/data/standard/03/04/30426.htm

[30] IULTCS/IUP 3. ISO 2419:2002 [IULTCS/IUP 1 ET IULTCS/IUP 3] Leather — Physical and mechanical tests — Sample preparation and conditioning [Internet]. ISO. 2002 [cited 2020 Feb 24]. Available from: https://www.iso.org/cms/render/live/en/sites/isoorg/contents/data/standard/02/40/24030.htm

[31] IULTCS/IUP 6. ISO 3376:2002 [IULTCS/IUP 6] Leather — Physical and mechanical tests — Determination of tensile strength and percentage extension [Internet]. ISO. 2002 [cited 2020 Feb 24]. Available from: https://www.iso.org/cms/render/live/en/sites/isoorg/contents/data/standard/02/40/24031.htm

[32] IULTCS/IUP 8. ISO3377-2:2002 [IULTCS/IUP 8] Leather — Physical and mechanical tests — Determination of tear load — Part 2: Double edge tear [Internet]. ISO. 2002 [cited 2019 Nov 4]. Available from: http://www.iso.org/cms/render/live/en/sites/isoorg/contents/data/standard/03/11/31148.html

[33] IULTCS/IUP 9. ISO 3379:2015 [IULTCS/IUP 9] Leather — Determination of distension and strength of surface (Ball burst method) [Internet]. ISO. 2015 [cited 2020 Feb 24]. Available from: https://www.iso.org/cms/render/live/en/sites/isoorg/contents/data/standard/06/38/63871.htm

[34] Eaton AD, Clesceri LS, Rice EW, Greenberg AE, Franson MAH. Standard methods for the examination of water and wastewater . Washington, DC: American Public Health Association (APHA); American Water Works Association; Water Environment Federation. 1995.

[35] Li S. Chapter 11 - Fundamentals of Biochemical Reaction Engineering. In: Li S, editor. Reaction Engineering [Internet]. Boston: Butterworth-Heinemann; 2017. p. 491–539. Available from: http://www.sciencedirect.com/science/article/pii/B9780124104167000112

[36] Reece JB, Urry LA, Cain ML, Wasserman SA, Minorsky PV, Jackson RB. Campbell Biology (10th Edition) [Internet]. New York: Pearson; 2013 [cited 2020 Jan 7]. Available from: https://www.biblio.com/book/campbell-biology-10th-edition-reece-jane/d/1290432081

[37] Deng A, Wu J, Zhang Y, Zhang G, Wen T. Purification and characterization of a surfactant-stable high-alkaline protease from Bacillus sp. B001. Bioresource technology. 2010;101(18):7100–7106. https://doi.org/10.1016/j.biortech.2010.03.130

[38] Dorra G, Ines K, Imen BS, Laurent C, Sana A, Olfa T, et al. Purification and characterization of a novel high molecular weight alkaline protease produced by an endophytic Bacillus halotolerans strain CT2. International journal of biological macromolecules. 2018; 111:342–351. https://doi.org/10.1016/j.ijbiomac.2018.01.024

[39] Gao R, Shi T, Liu X, Zhao M, Cui H, Yuan L. Purification and characterisation of a salt-stable protease from the halophilic archaeon Halogranum rubrum. Journal of the Science of Food and Agriculture. 2017;97(5):1412–1419. https://doi.org/10.1002/jsfa.7879

[40] Jaouadi B, Abdelmalek B, Fodil D, Ferradji FZ, Rekik H, Zaraî N, et al. Purification and characterization of a thermostable keratinolytic serine alkaline proteinase from Streptomyces sp. strain AB1 with high stability in organic solvents. Bioresource technology. 2010;101(21):8361–8369. https://doi.org/10.1016/j.biortech.2010.05.066

[41] Daniel RM, Danson MJ. Temperature and the catalytic activity of enzymes: A fresh understanding. FEBS Letters [Internet]. 2013 [cited 2019 Nov 13]; 587(17):2738–43. Available from: https://febs.onlinelibrary.wiley.com/doi/abs/10.1016/j.febslet.2013.06.027

[42] Covington AD. Tanning Chemistry: The Science of Leather. Royal Society of Chemistry; 2011. 483 p.

[43] Sheikh J, Bramhecha I. 6 - Enzymes for green chemical processing of cotton. In: Shahid-ul-Islam, Butola BS, editors. The Impact and Prospects of Green Chemistry for Textile Technology [Internet]. Woodhead Publishing; 2019 [cited 2019 Nov 14]. p. 135–60. (The Textile Institute Book Series). Available from: http://www.sciencedirect.com/science/article/pii/B978008102491100006X

[44] Nagal S, Kango N, Jain PC. Production of alkaline protease from Elizabethkingia meningoseptica KB042 using chicken feathers. Annals of Microbiology [Internet]. 2010 Dec [cited 2020 Apr 23];60(4):629–35. https://doi.org/10.1007/s13213-010-0101-9

[45] Vijayaraghavan P, Lazarus S, Vincent SGP. De-hairing protease production by an isolated Bacillus cereus strain AT under solid-state fermentation using cow dung: Biosynthesis and properties. Saudi journal of biological sciences. 2014;21(1):27–34. https://doi.org/10.1016/j.sjbs.2013.04.010

[46] Santos LM, Gutterres M. Reusing of a hide waste for leather fatliquoring. Journal of Cleaner Production. 2007;15(1):12–16. https://doi.org/10.1016/j.jclepro.2006.01.025

[47] Shaikh M, Shafique M, Naz SA, Jabeen N, Solangi BA. Streptomyces sp MM-3 from rhizosphere of psidium guajava: a potential candidate for protease with dehairing properties. Pakistan Journal of Botany [Internet]. 2018;51(2):735–42. Available from: http://inis.iaea.org/search/search.aspx?orig_q=RN:50037183

[48] Sivasubramanian S, Manohar BM, Puvanakrishnan R. Mechanism of enzymatic dehairing of skins using a bacterial alkaline protease. Chemosphere. 2008;70(6):1025–1034. https://doi.org/10.1016/j.chemosphere.2007.07.084

[49] Galarza BC, Cavello I, Greco CA, Hours R, Schuldt MM, Cantera CS. Alternative technologies for adding value to bovine hair waste. Journal of the Society of Leather Technologists and Chemists. 2010;94(1):26.

[50] Sivasubramanian S, Murali Manohar B, Rajaram A, Puvanakrishnan R. Ecofriendly lime and sulfide free enzymatic dehairing of skins and hides using a bacterial alkaline protease. Chemosphere [Internet]. 2008 Jan [cited 2019 Oct 9];70(6):1015–24. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0045653507011605

[51] Thanikaivelan P, Rao JR, Nair BU. Development of a leather processing method in narrow pH profile: part 1. Standardisation of dehairing process. Journal of the Society of Leather Technologists and Chemists. 2000;84(6):276–84.

[52] Thanikaivelan P, Rao JR, Nair BU, Ramasami T. Zero discharge tanning: a shift from chemical to biocatalytic leather processing. Environmental science & technology. 2002;36(19):4187–4194. https://doi.org/10.1021/es025618i

[53] Thanikaivelan P, Rao J, Nair B. Development of leather processing method in narrow pH profile: Part 2. Standardisation of tanning process. Journal- Society of Leather Technologists and Chemists. 2001;85:106–15.

[54] UNIDO. United Nations Industrial Development Organization: Acceptable quality standards in the leather and footwear industry. [Internet]. Vienna: United Nations Industrial Development Organization; 1996. 95 p. (General studies series.). Available from: //catalog.hathitrust.org/Record/101805463

[55] Nalyanya K, Rop R, Onyuka A, Kamau J. Tensile Properties of Indigenous Kenyan Boran Pickled and Tanned Bovine Hide. International Journal of Science and Research. 2015;438:2149–54.

[56] Nalyanya KM, Rop RK, Onyuka A, Birech Z, Sasia A. Effect of Crusting Operations on the Physical Properties of Leather. Leather and Footwear Journal [Internet]. 2018 Dec 28 [cited 2019 Oct 9];18(4):283–94. Available from: http://revistapielarieincaltaminte.ro/revistapielarieincaltaminteresurse/en/fisiere/full/vol18-nr4/article4_vol18_issue4.pdf

[57] Sizeland KH, Basil-Jones MM, Edmonds RL, Cooper SM, Kirby N, Hawley A, et al. Collagen orientation and leather strength for selected mammals. Journal of agricultural and food chemistry. 2013;61(4):887–892. https://doi.org/10.1021/jf3043067