European Journal of Sustainable Development Research
2023, 7(4), em0233
e-ISSN: 2542-4742
https://www.ejosdr.com

MODESTUM

Research Article

OPEN ACCESS

Enzymatic dehairing of sheep skin: Recovery and characterization of commercially important wool hydrolysate and fats

Sammy Kiplagat Chebon [1*], Wycliffe Chisutia Wanyonyi [2], John Mmari Onyari [1], Shital Mahindra Maru [3], Francis Jackim Mulaa [4]

[1] Department of Chemistry, Faculty of Science and Technology, University of Nairobi, Nairobi, KENYA
[2] Department of Mathematics, Actuarial and Physical Sciences, School of Science and Technology, University of Kabianga, Kericho, KENYA
[3] Department of Pharmacy, Faculty of Health Sciences, University of Nairobi, Nairobi, KENYA
[4] Department of Biochemistry, Faculty of Science and Technology, University of Nairobi, Nairobi, KENYA
*Corresponding Author: sammychebon88@gmail.com

Citation: Chebon, S. K., Wanyonyi, W. C., Onyari, J. M., Maru, S. M., & Mulaa, F. J. (2023). Enzymatic dehairing of sheep skin: Recovery and characterization of commercially important wool hydrolysate and fats. *European Journal of Sustainable Development Research, 7*(4), em0233. https://doi.org/10.29333/ejosdr/13501

ARTICLE INFO

Received: 12 Sep. 2022
Accepted: 05 Jul. 2023

ABSTRACT

Conventional dehairing methods in the traditional leather-making processes, consume large amounts of toxic chemicals and produce a toxic sludge/effluent, posing disposal challenges and consequently environmental pollution. The by-products of leather processing such as hair and fat, contain toxic chemicals. In this study, crude alkaline protease from *bacillus cereus strain 1-p,* was used to dehair sheepskin with up to 99.00% recovery of valuable wool and fat. The optimum temperature and pH for wool removal were found to be 30 ˚C and 11, respectively. The recovered wool was enzymatically hydrolyzed to obtain wool hydrolysate powder (48.1% yield). FTIR spectra of the wool hydrolysate showed the presence of amide A, I, and II absorption bands. Further, the amino acid analysis, revealed the presence of 15 amino acids, with glutamic acid (19.65 mole %), glycine (12.72 mole %), and leucine (9.42 mole %) being the most abundant. Fat was trans-esterified using methanol, in the presence of tert-butanol, and the resultant fatty acid methyl esters characterization was done using gas chromatography/mass spectrometry (GC/MS) analysis. GC/MS analysis showed the presence of 60 methyl esters corresponding to 60 fatty acids. The most abundant fatty acid was 9-octadecenoic (oleic) acid (41.64%), followed by hexa-decanoic (palmitic) acid (22.50%), and tetra-decanoic (myristic) acid (4.21%). Thermo-gravimetric analysis of the wool hydrolysate showed that it had good thermal stability. It is shown that crude alkaline protease extracted from *bacillus cereus strain 1-p* can completely eliminate the use of toxic sodium sulfide and lime in dehairing of skins/hide in tanneries, eliminating environmental pollution. Furthermore, the recovery of fat and wool using an eco-friendly enzymatic dehairing process can significantly reduce the pollution load in the effluent. The recovered wool and fats can be applied in the production of wool hydrolysate, which is high in protein content, amino acids and biodiesel.

Keywords: enzymes, wool removal, amino acids, fatty acid methyl esters

INTRODUCTION

The leather industry plays an important role in the global economy, with an approximate annual trade value of US$394.12 billion and a compounded annual growth rate of 5.90% (Muralidharan et al., 2022). In developing countries such as Tanzania, Ethiopia, and Kenya, the industry greatly contributes to the growth of economies through employment, enhanced gross domestic product (GDP), and enhanced gross national income (GNI) (Ani & Onu, 2022; Dvouletỳ et al., 2018). Some of the commercial leather products include shoes, leather clothing, automotive interiors, book coverings, pet accessories, hunting accessories, tent coverings, boots, belts, boat coverings, balls, music items, knife sheaths, lacing, purses, gloves, luggage bags, coats, aircraft, furniture and other leather goods (Wanyonyi & Mulaa, 2019).

Despite its paramount contribution to socio-economic development, the leather industry is often condemned for environmental degradation due to extensive chemical use and the generation of hazardous waste. The industry has indeed been ranked among the most polluting ones (Kanagaraj et al., 2016; Yorgancioglu et al., 2020). Therefore, effective and costly effluent treatment prior to disposal is necessary (Mutunga et al., 2020).

Dehairing is a critical step in the leather production process. Conventional leather manufacturing processes utilizes huge quantities of toxic chemicals (lime and sodium sulfide) in the dehairing stage. It is estimated that dehairing

alone contributes between 60-70% of the total pollution load of the leather production process in terms of 50%-70% of the total chemical oxygen demand (COD), biochemical oxygen demand (BOD), as well as 15%-20% total solids (TS) in tannery waste (Saranya et al., 2016). In addition, the toxic chemicals contaminate fats and destroy hair/wool to levels where recovery and recycling is difficult. Harmful sludge and obnoxious odor gases such as hydrogen sulfide are generated in huge quantities resulting in air, water and soil pollution (Kanagaraj et al., 2016). Consequently, there is need to adopt eco-friendly leather processing methods for sustainable environmental conservation.

Environmentally friendly dehairing methods, where enzymes are used as substitutes for the toxic sodium sulfide has attracted increased attention (Wanyonyi et al., 2016). Unlike the conventional chemical dehairing methods, enzymatic dehairing techniques are attractive since they are ecofriendly, yield high quality leather, and allow recovery of fats and hair/wool in good conditions for recycling. Additionally, no hydrogen sulfide is produced during dehairing process. Such methods are environmentally friendly, safer and they mitigate environmental pollution. Alkaline proteases have been shown to be very effective in removal of hair/wool from hide and skins without damaging the hair/wool. A protease extracted from alkaliphilic *idiomarina sp. C9-1* completely dehaired cattle hide as well as rabbit, pig, and goat skins within eight to 12 hours, without using sodium sulfide. There was no damage to the grain surfaces and the good condition of hair allowed for recycling (Berechet et al., 2018; Zhou et al., 2018).

Even though the new dehairing methods are relatively safe and effective, pollution still occurs, to some extent, if the recovered fats and hair/wool are not managed well. Therefore, recycling hair/wool and fats can reduce the environmental pollution burden and also increase the income for farmers and tanneries. Hydrolysis of the hair/wool to generate wool keratin hydrolysate is a good example of waste valorization, since keratin hydrolysate has a wide range of industrial applications. Keratin hydrolysate powder can be used to prepare livestock feed, fire extinguisher foaming agents, biodegradable films, slow-release fertilizer, glues, enhance pesticide and water retention, and amend degraded soils (Chojnacka et al., 2011; Gaidau et al., 2019).

Further, biodiesel prepared from renewable resources has received increased attention as an alternative energy source to fossil fuels that are not from renewable sources (Osawa et al., 2014, 2015). Whenever fossil fuels are burnt, greenhouse gases (GHGs) are produced. These gases are known to cause climate change via heat entrapment in the atmosphere. Biodiesel has a lower carbon footprint and has been shown to lower greenhouse gas emissions to the atmosphere hence a viable solution to address climate change problems. Biodiesel is produced via trans-esterification of lipids (fats or oils). The fat collected from sheep skin dehairing process can subsequently undergo trans-esterification to obtain biodiesel, that can be used by farmers, and industry.

The present study sought to dehair sheepskin using a crude alkaline protease from *bacillus cereus strain 1-p*. Factors affecting the dehairing process such as temperature and pH were examined. The resultant wool was further enzymatically hydrolyzed and characterized using Fourier Transform Infrared (FTIR) spectroscopy, amino acid analysis, and thermo-gravimetric analysis (TGA). Similarly, the recovered fats were trans-esterified, using methanol, in the presence of tert-butanol, to produce fatty acid methyl esters (biodiesel) and characterized using GC-MS. The findings in this study will provide a rational and scalable solution for enzymatic leather processing with broader benefits to environmental conservation, energy, food security, and bio-renewable waste recycling.

METHODOLOGY

Materials

Sheepskins were purchased from “Kiamaiko”, a local slaughterhouse in Nairobi, Kenya. All chemicals, reagents, culture media, organic and inorganic compounds were bought from Sigma-Aldrich and were of analytical grade.

Enzyme Production

Crude alkaline protease was produced as outlined by Wanyonyi et al. (2014), with slight modifications, i.e., the use of a bioreactor and slightly varying salt quantities in the media. Media containing 15.0 g casein, 6.0 g K_2HPO_4, 4.5 g KH_2PO_4, 0.9 g urea, 0.9 g $MgSO_4.7H_2O$, 0.9g $CaCl_2$, 7.5 g glucose, 1.5 g yeast extract, and three liters of distilled water was prepared. The pH was adjusted to 11.5, sterilized in an autoclave (Tuttnauer steam sterilizer) at 121 ºC for 30 minutes and put in the bioreactor (R’ALF plus duet fermenter, 3.7L) vessel. The media was inoculated with overnight grown bacterial culture containing *bacillus cereus strain 1-p* at optimum condition for 72 hours. The resultant enzyme was centrifuged at 10,000 rotations per minute, for 15 minutes, and stored at 4 °C for future use.

Determination of Optimum pH for Wool Removal

Thirty six (36) identical pieces of sheepskin each measuring 10 cm by 10 cm were cut from sheepskin and thoroughly washed with tap water to get rid of dirt and soluble impurities. The pieces were subsequently soaked in water for 1 hour to increase their flexibility and swelling. 250 ml of fresh crude enzyme was placed in 12 different 500 ml conical flasks, whose pH had been pre-adjusted to pH values varying from 2 to 13. A piece of skin was separately immersed in each conical flask and placed on a thermolyne orbital shaker at 200 rpm at 22 °C. Subsequently, the skin pieces were removed from the conical flasks, at every one-hour time intervals and the extent of wool removal analyzed. Since all the pieces were almost identical, the average weight of all wool removed (100%) from one piece was first determined, which represented 100% area removal. The total area of un-dehaired portion was determined by subtracting the weight of wool removed at a particular time (T) from the value of 100% wool removed. All experiments were conducted in duplicate, and the average values reported. For each pH analyzed, control experiments were done under the same pH and temperature, but the enzyme solution was substituted with distilled water. Any enzyme remaining on the tray after the analysis was poured back into the conical flask and the piece of skin was returned. The percentage of removal

was determined as per the formula. Percent removal (%)=(*A'*/*A*)×100, where *A'* is the area of the skin piece whose hair has been removed off and *A* is the total are of the skin piece.

Determination of Optimum Temperature for Wool Removal

Nine pieces of sheepskin each measuring 10 cm by 10 cm were cut and thoroughly cleaned with water to get rid of dirt and soluble impurities. 250 ml of freshly prepared enzyme was put in 500 ml conical flasks and pH adjusted to the optimum pH established in the previous section. Each piece was immersed in the enzyme solutions and put on thermolyne orbital shaker at 200 rpm set at different temperatures (25 °C, 30 °C, and 35 °C). The experiments were conducted in duplicate and an average value reported. Similarly, control experiments were done under the same pH and temperature, but the enzyme solution was substituted with distilled water. A piece was removed from the conical flask at one hour time intervals, and the extent of wool removal examined. After analysis, any enzyme remaining on the tray and the piece of skin was returned back into the conical flask. The percentage of removal was determined as per the formula. Percent removal (%)=(A'/A)×100, where *A'* is the area of the skin piece whose hair has been removed off and *A* is the total area of the skin piece.

Wool Hydrolysis

After determination of the optimum conditions for the dehairing process, a full sheepskin was soaked in a bucket filled with a solution of the crude alkaline protease enzyme from *bacillus cereus strain 1-p* at pH 11.0 and incubated at temperature of 30 °C in an orbital shaker at 200 rpm for four hours. The skin weight (kg) to enzyme volume (L) ratio was 1:1 (w/v). Once the wool had loosened out, it was gently removed by scraping using a blunt knife. The removed wool was recovered by filtering the enzyme liquor before thorough washing and sundried to constant weight. Similarly, fats were recovered by decanting from the enzyme liquor followed by washing to remove any enzyme or residue remaining. 2.5 L of freshly prepared alkaline protease solution at pH 11 was placed in a 5.0 L conical flask before adding 287 g of wool. The content was placed in an oven set at 75 ºC. The pH of the set up was adjusted periodically in order to ensure that it remained at the optimum value. Once the hydrolysis was complete, the solution was freeze dried to obtain a powder hydrolysate. The yield of wool hydrolysate was calculated using the formula: Yield (%)=(W'/W)×100, where *W'* is the weight of the hydrolysate obtained after freeze drying and *W* is the weight of the original wool.

Fourier Transform Infrared Spectroscopy

Fourier transform infrared spectrophotometer (IRAffinity-1S FTIR Shimadzu model) was used to identify functional groups on wool hydrolysate at a frequency range of 500 to 4,000 cm^{-1}. A horizontal ATR sampling accessory was used, and the crystal wiped clean prior to any reading. A background spectrum was developed when the accessory was empty. Samples were placed on the accessory and positioned in a way that there was good contact with the crystal and the acquisition of spectra done.

Thermogravimetric Analysis

Thermogravimetric analysis of the wool hydrolysate was done on a Mettler Tolledo TGA/DSC 3+ (Mettler-Toledo GmbH, Switzerland). The analysis was done at temperature range of 25 °C to 600 °C and at a heating rate of 20 °C/min, under nitrogen gas atmosphere. The mass of the sample used was approximately 10 mg.

Amino Acid Analysis

A given mass (about 10 mg) of the sample was transferred into a glass hydrolysis tube in which it was dried. Liquid phase hydrolysis (200 µl 6N HCl and 1% phenol) was done at 110 °C for 24 hours. The tube was then cooled and unsealed so as to allow the sample to dry. Once dry, the sample was dissolved in Pickering Na buffer solution and sodium diluent added together with NorLeucine as the internal standard. 50 µL of the sample was injected into a concise ion exchange column. The column eluent was reacted with ninhydrin for detection. Response factors were determined using amino acids standards solution for protein hydrolysate on Na-based Hitachi 8,800 amino acids analyzer after calibrating the analyzer for all amino acids.

Trans-Esterification

The earlier collected fats were washed in distilled water and dissolved in hexane. Hexane was then evaporated to obtain solid fat. The trans-esterification (fat; methanol molar ratio 1:25) using methanol-tert-butanol was done in order to analyze fat content. 1 gram of fat was loaded in falcon tubes and fixed on a shaker and transferred to an oven set at 45 ºC. When the fat was completely molten, a mixture of 4 ml of methanol and 1 ml of tert-butanol previously mixed in a separate tube was added. 0.1 grams of lipase enzyme (novozyme 435) were added in the tube. After 24-hour intervals, thin layer chromatography analysis of the samples was carried out on a silica gel plate so as to ascertain the presence of trans-esterification products. The mobile phase was hexane: diethyl ether in a 90:10 ratio. Coloration was done in an iodine chamber.

Gas Chromatography/Mass Spectrometry Analysis

Once the thin layer chromatography showed there was complete trans-esterification, samples were taken through gas chromatography/mass spectrometry (GC-MS) analysis. The Falcone tubes were vortexed for 10 seconds prior to 15-minute sonication and centrifugation. 1 ml of the top layer (supernatant) was picked and dried over anhydrous sodium sulphate (Na_2SO_4) before analysis by GC-FID. 1,000× dilution of the samples in hexane was carried out and analysis done on an agilent gas chromatograph 7890A/5975 C mass spectrometer in full scan mode equipped with integrated gas chromatograph with a HP-5 MS low bleed capillary column (30 m×0.25 mm, internal diameter 0.25 µm). Helium was used as carrier gas at a flow rate of 1.25 mL/min. The initial column temperature was held at 35 °C for five minutes, then increased linearly at 10 °C/min to 280 °C, followed by 50°C/min to 285 °C. The ionization was carried out in the electron impact mode. The electron multiplier voltage (El 70 eV) and automatic gain control target were set automatically.

RESULTS AND DISCUSSION

Effect of pH on Dehairing of Sheepskin

The pH plays a significant role in enzyme reactions. The effect of pH on dehairing of sheep skin by crude alkaline protease extracted from *bacillus cereus strain 1-p* was investigated at a pH range of 2 to 13.

As shown in **Figure 1**, an increase in pH, resulted in an increased wool removal. The highest wool removal was witnessed in alkaline range with over 85% removal at pH 9 after eight hours. However, 100% wool removal was achieved after six and five hours at pH 11 and pH 12, respectively.

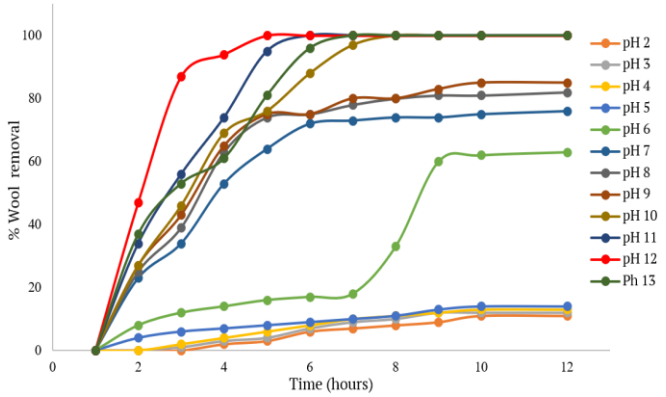

Figure 1. Effect of pH on wool removal using crude alkaline protease (Source: Authors' own elaboration)

In order to achieve high quality leather, a very high pH is not desirable since it may destroy skin grains. As a result, this may deteriorate the quality of leather and also hinder wool recyling by reducing its strength properties. The optimum pH for wool removal using alkaline protease from *bacillus cereus strain 1-p* was therefore chosen to be 11. Al-Khaldi and Al-Abdalall (2017) reported that an alkaline protease, hydrolyzes hair root proteins, thus facilitating easy removal of the hair. The particular substances that are degraded are proteoglycans and glycoproteins, which are found in the basal membrane. Degradation of the proteoglycans results in detachment of collagen fibril bundles as well as fibrils from collagen fibers, which results in swelling. The function of glycoproteins is to hold hair follicles in the skin's extracellular matrix. When the glycoproteins are degraded, the skin hairs become dislodged enabling dehairing (Sharma et al., 2016; Wang et al., 2020; Zambare et al., 2011).

Control experiments for each pH were also done and the results obtained are presented in **Figure 2**. At low acidic pH, the percent hair removal was very low.

As shown in **Figure 2**, relatively low wool removal (%) was observed for the control after six hrs at pH 11 and 12, compared to results obtained with the use of the enzyme (**Figure 1**). As the pH increased, the percent hair removal increased gradually up to pH 13. Notably, no complete wool removal was observed at all pH tested without use of enzyme. In this study, it is shown that the use of crude alkaline protease resulted in complete wool removal. A high alkaline pH is known to open up collagen fiber structure through cleaving of skin tissue's interfibrillar and therefore swelling the skin. Under such

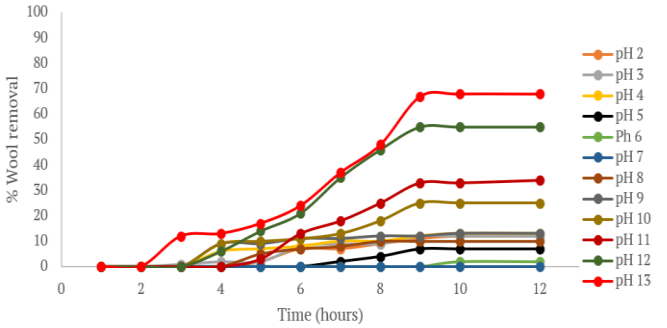

Figure 2. Effect of pH on wool removal, in distilled water (control) (Source: Authors' own elaboration)

conditions, some of the hair or wool may come off (Van Der Ham, 2021; Sivasubramanian et al., 2008).

Effect of Temperature on Dehairing of Sheepskin

Temperature also plays a critical role with regard to the activity of an enzyme (Grahame et al., 2015). High temperatures cause skin degradation and deterioration of leather quality. In this study, the temperature was varied from 22 ºC, and 35 ºC, as recommended in the literature (Covington & Wise, 2019; Nyakundi et al., 2021). The effectof temperature on wool removal by the crude protease at the optimum pH was evaluated at 22 ºC, 25 ºC, 30 ºC, and 35ºC (**Figure 3**).

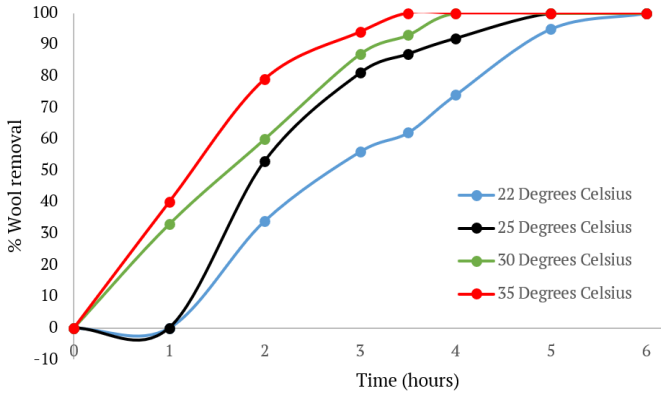

Figure 3. Effect of temperature on wool removal (Source: Authors' own elaboration)

The results show that as the temperature increased, the enzyme activity increased. In all the experiments, complete wool removal was achieved. Complete wool removal took the shortest time (3.5 hours) at 35 ºC followed by 30 ºC (four hours), 25 ºC (five hours), and 22 ºC (six hours). At neutral pH, raw skins have a shrinkage/denaturation temperature of around 65 °C. Under highly alkaline pH, the shrinkage temperature is 50 °C or even lower. The recommended dehairing temperature is therefore 30 ºC or less (Covington & Wise, 2019; Nyakundi et al., 2021). The optimal temperature of 30 ºC, was therefore adopted for maximum protease activity for the enzyme derived from *bacillus cereus strain 1-p*. Similar findings have previously been reported in the dehairing of animal hides and skins by alkaline proteases of aspergillus oryzae (Never et al., 2019).

Enzymatic Dehairing of Sheepskin and Wool Hydrolysis Yield

Complete dehairing of 1.67 kg sheepskin yielded 356 g of dry wool. 287 g of the recovered wool was subjected to enzymatic hydrolysis, using alkaline protease from *bacillus cereus strain 1-p*. After complete hydrolysis and freeze drying, 137.99 g of wool hydrolysate powder was obtained corresponding to a yield of 48.08%. The yield is better than what has been reported in chemical hydrolysis methods in another study, which ranged from 16.40% to 43.50% (Berechet et al., 2018). Enzymatic hydrolysis is thus a better technique, since higher yields were obtained, and it is eco-friendly compared to the chemical hydrolysis method.

FTIR Analysis

FTIR analysis was done to establish the key functional group present on wool and wool hydrolysate powder. As shown in **Figure 4**, bands corresponding to amide A (3265.5 cm-1), C-H stretching bonds (2973.7cm-1), amide I (1650 cm-1), and amide II (1537.3 cm-1) were observed. The broad bands observed between 3,500 cm-1–3,200 cm-1 are associated with stretching vibration of –O-H and –N-H (amide A). Bands obtained between 3,000-2,800 cm-1 are attributed to C-H stretching bonds while those at 1,700-1,600 cm-1 are due to CO stretching vibration (amide I). Amide II bands due to N-H bending vibration and C-N stretching vibration occur at 1,580 -1,480 cm-1 (Gaidau et al., 2019). The band at 995.3 cm-1 was absent in the wool spectrum. It is associated with the presence of more oxidized groups. It also explains the darker color of the hydrolysate (Gaidau et al., 2021). Also absent in the wool spectrum is the band at 1116.67 cm-1, further demonstrating the difference difference between wool and its hydrolysate. Similar observations have been reported for wool hydrolysates (Gaidau et al., 2019; Prayitno et al., 2017) and thus confirming that the wool hydrolysate contains proteins. It would therefore be interesting to determine other parameters and further evaluate possible use of the wool keratin hydrolysate as an additive for livestock feed preparation.

Amino Acid Analysis

An amino acid analysis of the resultant wool hydrolysate powder was used to determine the composition of the keratin hydrolysate obtained and the results are shown in **Figure 5** and **Table 1**. 15 amino acid were detected in the sample.

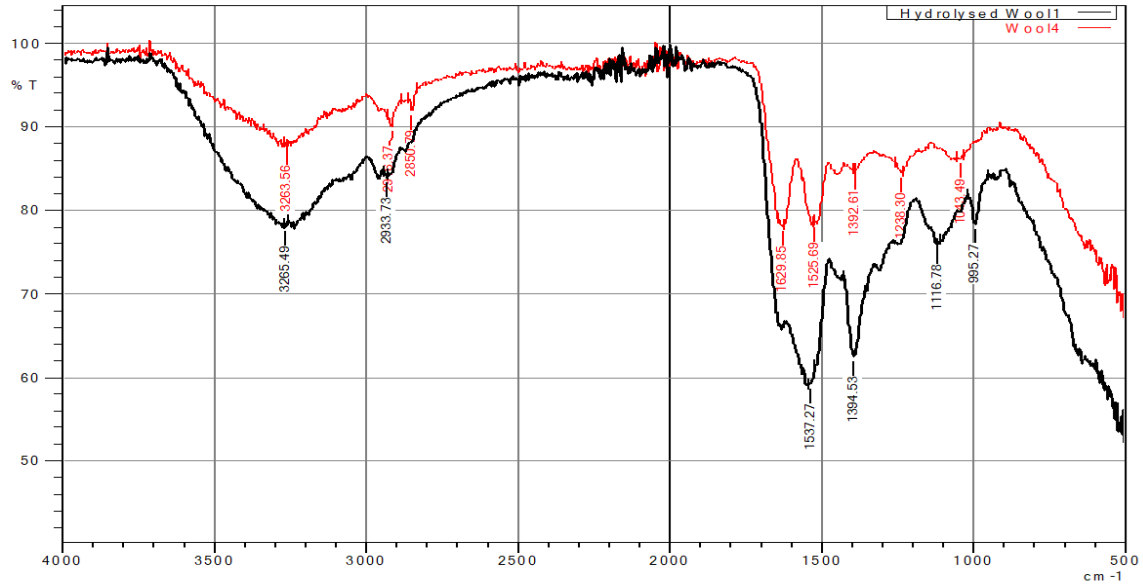

Figure 4. Fourier transform infrared spectra of wool & wool hydrolysate (Source: Authors' own elaboration)

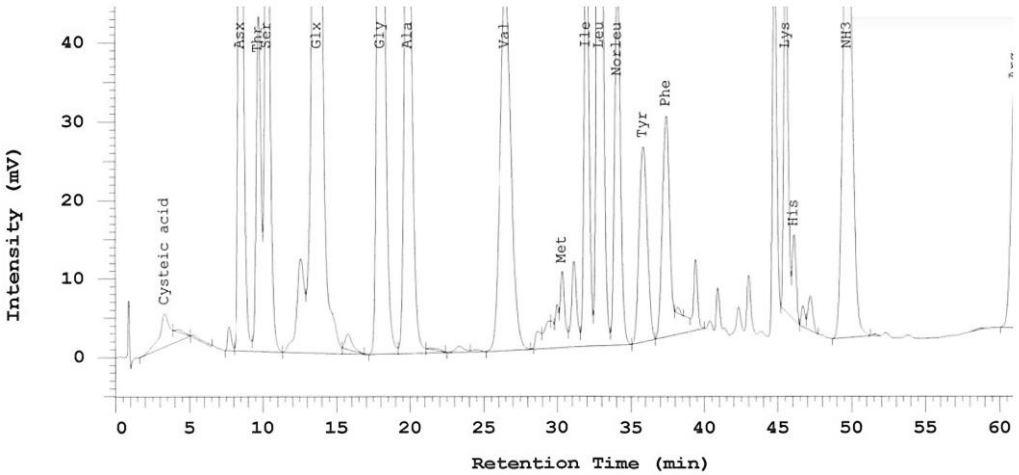

Figure 5. Amino acids chromatogram obtained using a Hitachi 8800 amino acids analyzer (Source: Authors' own elaboration)

Table 1. Amino acid composition of wool hydrolysate

Amino acid	Mole %	Weight %
Aspartic acid	8.11	8.76
Threonine	2.97	2.81
Serine	5.57	4.56
Glutamic acid	19.65	23.81
Proline	8.62	7.86
Glycine	12.72	6.82
Alanine	8.33	5.56
Valine	7.32	6.81
Isoleucine	3.69	3.92
Leucine	9.42	10.01
Tyrosine	3.12	4.78
Phenylalanine	3.21	4.44
Histidine	0.56	0.72
Lysine	2.66	3.20
Arginine	4.05	5.94

The most abundant was glutamic acid (19.65 mole %) followed by glycine (12.72 mole %) and leucine (9.42 mole %). The results obtained in our study agree with other research findings, which also observed that glutamic acid is the most abundant amino acid in keratin hydrolysates (Rajabinejad et al., 2018; Tsuda & Nomura, 2014).

The presence of essential amino acids in the wool hydrolysate is significant in terms of its nutritional value in formulation of animal feeds. Essential amino acids are those amino acids that vertebrates cannot synthesize and thus must be supplied from the diet (Lopez & Mohiuddin, 2020). Such amino acids that are present in the wool hydrolysate include leucine (9.42 mole %), valine (7.32 mole %), and phenylalanine (3.21 mole %). In addition glutamate, phenylalanine, cysteine, and glycine can be applied as foliar or seed fertilizers, to increase productivity of legumes to about 22% (Teixeira et al., 2018).

Trans-Esterification and Characterization

The thin layer chromatography (TLC) results are displayed in **Figure 6**. Spot B is the trans-esterified product and spot B-, is the negative control (untreated sheep fat). In B, there is a spot at the top that is absent from B-. This suggests the fat was trans-esterified to fatty acid methyl esters (biodiesel). There is also a spot in B- that is absent in B, and lower than the earlier mentioned spot in B. The spot in B- represents triacyl glycerides that will be transformed to fatty methyl esters shown in B. The presence of tert-butanol facilitates complete trans-esterification. This can be attributed to the fact that tert-butanol enhances the substrate's (sheep skin fat in this case) solubility in methanol. The increased solubility means there is less free methanol, which is known to cause inhibition of the lipase. Tert-butanol also minimizes the negative effects of associated any excess methanol. (Azócar et al., 2014; Kumari et al., 2009).

Figure 6. TLC plate after trans-esterification (B: Trans esterified fat & B-: Negative control) (Source: Authors' own elaboration)

Further, GC-MS analysis of the sheep oil fat obtained from the enzyme hydrolysis reaction showed the presence of 61 fatty acid methyl esters as shown in **Table 2**.

Since biodiesel is a combination of fatty acid methyl esters (products of the reaction between methanol and fatty acids),

Table 2. GC-MS analysis of sheepskin fat

Retention time (min)	Library/ID	Conc ng/µl	Abundance (%)
5.09	Butanoic acid, 3,3-dimethyl-	0.02	0.00
9.71	Hexanoic acid, methyl ester	0.60	0.04
10.63	Formic acid, heptyl ester	0.54	0.04
11.63	Heptanoic acid, methyl ester	2.58	0.19
13.36	Methyl octanoate	5.92	0.44
14.90	Methyl nonanoate	1.00	0.07
15.62	Pentadecanoic acid	0.30	0.02
15.98	4-Decenoic acid, methyl ester	0.38	0.03
16.11	Fumaric acid, neopentyl undecyl ester	0.05	0.00
16.22	Methyl 6-methyloctanoate	0.10	0.01
16.34	Methyl decanoate	3.23	0.24
16.47	Methyl 8-oxooctanoate	0.84	0.06
17.66	Methyl undecanoate	0.27	0.02
17.84	Nonanoic acid, 9-oxo-, methyl ester	1.90	0.14
17.97	Octanedioic acid, dimethyl ester	1.25	0.09
18.49	Undecanoic acid, 10-methyl-, methyl ester	0.05	0.00
18.93	Dodecanoic acid, methyl ester	4.45	0.33
19.09	10-Oxodecanoic acid, methyl ester	0.31	0.02
19.23	Nonanedioic acid, dimethyl ester	4.42	0.33
19.54	Dodecanoic acid, 4-methyl-, methyl ester	1.56	0.12
20.10	Tridecanoic acid, methyl ester	1.32	0.10

Table 2 (Continued). GC-MS analysis of sheepskin fat

Retention time (min)	Library/ID	Conc ng/µl	Abundance (%)
20.30	Decanoic acid, methyl ester	0.60	0.04
20.41	Decanedioic acid, dimethyl ester	0.43	0.03
20.68	Methyl 4,8-dimethylnonanoate	0.39	0.03
20.84	Tridecanoic acid, 12-methyl-, methyl ester	6.03	0.45
21.13	Methyl myristoleate	4.24	0.32
21.31	Methyl tetradecanoate	56.28	4.21
21.44	10-hydroxydecanoic acid, methyl ester	0.17	0.01
21.53	Undecanedioic acid, dimethyl ester	0.83	0.06
21.73	Methyl 8-methyl-decanoate	2.80	0.21
21.80	Tridecanoic acid, 4,8,12-trimethyl-, methyl ester	10.79	0.81
21.94	Methyl 13-methyltetradecanoate	8.44	0.63
22.05	Tetradecanoic acid, 12-methyl-, methyl ester	10.09	0.76
22.16	Cis-5-dodecenoic acid, methyl ester	1.72	0.13
22.34	Pentadecanoic acid, methyl ester	19.15	1.43
22.59	Dodecanedioic acid, dimethyl ester	0.91	0.07
22.67	Pentadecanoic acid, 13-methyl-, methyl ester	0.22	0.02
22.76	Undecanoic acid, methyl ester	1.42	0.11
22.99	Pentadecanoic acid, 14-methyl-, methyl ester	9.27	0.69
23.21	Methyl hexadec-9-enoate	55.57	4.16
23.59	Methyl palmitate (Methyl hexadecanoate)	300.53	22.50
23.82	Methyl 7-methylhexadecanoate	7.69	0.58
23.91	Methyl 10-methyl-hexadecanoate	10.88	0.81
24.02	Hexadecanoic acid, 15-methyl-, methyl ester	21.49	1.61
24.13	Hexadecanoic acid, 14-methyl-, methyl ester	50.58	3.79
24.40	Heptadecanoic acid, methyl ester	47.45	3.55
24.78	8-octadecenoic acid, methyl ester	3.84	0.29
25.30	9-octadecenoic acid, methyl ester, (E)-	556.29	41.64
25.86	Octadecanoic acid, 14-methyl-, methyl ester	5.29	0.40
25.94	Methyl 10-trans,12-cis-octadecadienoate	5.86	0.44
26.03	Cis-10-nonadecenoic acid, methyl ester	16.18	1.21
26.24	Nonadecanoic acid, methyl ester	11.34	0.85
26.71	Oxiraneoctanoic acid, 3-octyl-, methyl ester, cis-	39.79	2.98
27.06	Methyl 18-methylnonadecanoate	14.62	1.09
27.51	Cyclopropaneoctanoic acid, 2-octyl-, methyl ester, cis-	4.42	0.33
28.63	Docosanoic acid, methyl ester	2.59	0.19
29.39	Tricosanoic acid, methyl ester	2.14	0.16
29.95	9-octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester	8.91	0.67
30.13	Octadecanoic acid, 2,3-dihydroxypropyl ester	5.19	0.39
32.13	Hexacosanoic acid, methyl ester	0.21	0.02
40.17	9-octadecenoic acid (Z)-, 2-hydroxyethyl ester	0.10	0.01

sheep skin fat is very promising as a biodiesel source. It is demonstrated that the enzyme hydrolyzed fat (triacyl glyceride) produced glycerol and fatty acids. The amount of fatty acid methyl ester from a particular fatty acid produced directly reflects the amount of that fatty acid. Therefore, the most abundant fatty acid in sheepskin fat is 9-octadecenoic acid (41.64%), followed by hexa-decanoic acid (22.50%), and hexadec-9-enoic acid (4.16%). It is evidently clear that enzymatic dehairing of skin makes it possible for fat recovery without contamination. Recyling the recovered fat into biodiesel could be promising application of the recovered fat since they contain high concentration of 9-octadecenoic acid. The higher the amount of 9-octadecenoic acid in biodiesel, the lower the smoke emission from engines. Evidently, environmental conservation will be enhanced (Colak et al., 2005; Dinesha et al., 2018; Fonseca et al., 2019).

Thermogravimetric Analysis of Wool Hydrolysate Powder

A thermally stable compound can retain its strength, toughness, or elasticity even when heated (Król-Morkisz & Pielichowska, 2019), and can therefore be applied in a wide range of fields. TGA thermogram of wool hydrolysate powder is shown in **Figure 7**.

Two zones were evident. The first zone occurred between 30 °C - 125 °C and approximately 7% weight loss occurred. This

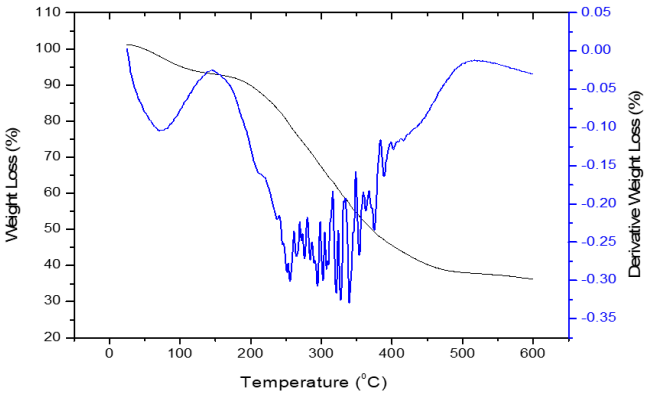

Figure 7. Thermogravimetric analysis of wool hydrolysate (Source: Authors' own elaboration)

zone can be attributed to loss of water. The second zone occurred from around 125 ºC - 490 ºC. This is due to thermal decomposition of the wool hydrolysate. The results also show that the weight loss did not get to 100% even at 600 ºC thus indicating that the wool hydrolysate has good thermal stability as reported in the literature (Rajabinejad et al., 2018). It is evident that the curve is also not very steep, demonstrating the thermal stability of the wool hydrolysate. TGA results suggest that wool hydrolysate powder could possibly be applied in wound healing as part of a drug delivery system. One of the requirements of a delivery system is thermal stability (Harmaen et al., 2016; Mohamed et al., 2022), as well as biocompatibility and biodegradability.

CONCLUSIONS

The current study has clearly demonstrated that crude alkaline protease from indigenous *bacillus cereus strain 1-p* can effectively and safely dehair sheepskin. Enzymatic dehairing, offers a timely solution to conventional chemical methods, which pose great danger to the environment due to extensive use of toxic chemicals. The recovered by-products (wool and fat) are non-toxic and can be used in a wide variety of applications. For instance, the crude alkaline protease that was used also displayed a remarkable ability to hydrolyze wool into wool hydrolysate powder rich in amino acid for application in animal feeds pharmaceuticals, and fertilizers formulations. The recovered fat was trans-esterified and GC-MS analysis confirmed the presence of 60 different fatty acid methyl esters, and by extension fatty acids, in sheepskin fat indicating that it could be used as raw material for biodiesel production. TGA results showed that wool hydrolysate has good thermal stability. The unique ability of the enzyme to dehair skin at a broad range of pH and biodegrade wool suggested that the enzyme could be useful in leather manufacturing and bioremediation solid tannery waste. Due to the crude nature of the enzyme used in this work, a large volume of it had to be used. If it was pure, a smaller amount would be necessary. Future research should thus aim at purification of the enzyme.

Author contributions: All co-authors have been involved in all stages of this study while preparing the final version. They all agree with the results and conclusions.

Funding: No funding source is reported for this study.

Acknowledgements: The authors would like to thank National Research Fund (NRF) Kenya and Bioinnovate Africa for their financial support, which enabled this work to be done efficiently and in a timely manner. The authors would also like to thank the Department of Chemistry and Department of Biochemistry at the University of Nairobi for provision of all infrastructure and facilities for the work.

Declaration of interest: No conflict of interest is declared by the authors.

Ethical statement: Authors stated that the study did not require ethics committee approval since it was in vitro.

Data sharing statement: Data supporting the findings and conclusions are available upon request from corresponding author.

REFERENCES

Al-Khaldi, E. M., & Al-Abdalall, A. H. (2017). Effect of activators and inhibitors on extracellular thermostable alkaline protease isolated from bacillus subtilis obtained from eastern province of Saudi Arabia. *African Journal of Biotechnology, 16*(5), 232-237. https://doi.org/10.5897/AJB2016.15429

Ani, K. J., & Onu, C. (2022). Effect of foreign direct investment on gross national income in Nigeria, 2006-2017. Independent Journal of Management & Production, 13(1), 056-071. https://doi.org/10.14807/ijmp.v13i1.1484

Azócar, L., Navia, R., Beroiz, L., Jeison, D., & Ciudad, G. (2014). Enzymatic biodiesel production kinetics using co-solvent and an anhydrous medium: A strategy to improve lipase performance in a semi-continuous reactor. *New Biotechnology, 31*(5), 422-429. https://doi.org/10.1016/j.nbt.2014.04.006

Berechet, M. D., Niculescu, M., Gaidau, C., Ignat, M., & Epure, D. G. (2018). Alkaline-enzymatic hydrolyses of wool waste for different applications. *Revista de Chimie-Bucharest* [*Bucharest Chemistry Magazine*], *69*(7), 1649-1654. https://doi.org/10.37358/RC.18.7.6388

Chojnacka, K., Górecka, H., Michalak, I., & Górecki, H. (2011). A review: valorization of keratinous materials. *Waste Biomass Valorization, 2*, 317-321. https://doi.org/10.1007/s12649-011-9074-6

Colak, S., Zengin, G., Ozgunay, H., Sarikahya, H., Sari, O., & Yuceer, L. (2005). Utilization of leather industry prefleshings in biodiesel production. *Journal of the American Leather Chemists Association, 100*(4), 137-141.

Covington, A. D., & Wise, W. R. (2019). *Tanning chemistry: The science of leather*. Royal Society of Chemistry. https://doi.org/10.1039/9781839168826

Dinesha, P., Jagannath, K., & Mohanan, P. (2018). Effect of varying 9-octadecenoic acid (oleic fatty acid) content in biofuel on the performance and emission of a compression ignition engine at varying compression ratio. *Biofuels, 9*(4), 441-448. https://doi.org/10.1080/17597269.2016.1275491

Dvouletỳ, O., Gordievskaya, A., & Procházka, D. A. (2018). Investigating the relationship between entrepreneurship and regional development: Case of developing countries. *Journal of Global Entrepreneurship Research, 8*, 16. https://doi.org/10.1186/s40497-018-0103-9

Fonseca, J. M., Teleken, J. G., de Cinque Almeida, V., & da Silva, C. (2019). Biodiesel from waste frying oils: Methods of production and purification. *Energy Conversion and Management, 184*, 205-218. https://doi.org/10.1016/j.enconman.2019.01.061

Gaidau, C., Epure, D.-G., Enascuta, C. E., Carsote, C., Sendrea, C., Proietti, N., Chen, W., & Gu, H. (2019). Wool keratin total solubilisation for recovery and reintegration–An ecological approach. *Journal of Cleaner Production, 236*, 117586. https://doi.org/10.1016/j.jclepro.2019.07.061

Gaidau, C., Stanca, M., Niculescu, M.-D., Alexe, C.-A., Becheritu, M., Horoias, R., Cioineag, C., Râpă, M., & Stanculescu, I. R. (2021). Wool keratin hydrolysates for bioactive additives preparation. *Materials, 14*, 4696. https://doi.org/10.3390/ma14164696

Grahame, D. A. S., Bryksa, B. C., & Yada, R. Y. (2015). Factors affecting enzyme activity. In R. Y. Yada (Ed.), *Improving and tailoring enzymes for food quality and functionality* (pp. 11-55). Elsevier. https://doi.org/10.1016/B978-1-78242-285-3.00002-8

Harmaen, A. S., Khalina, A., Ali, H. M., & Azowa, I. N. (2016). Thermal, morphological, and biodegradability properties of bioplastic fertilizer composites made of oil palm biomass, fertilizer, and poly(hydroxybutyrate-co-valerate). *International Journal of Polymer Science, 2016*, e3230109. https://doi.org/10.1155/2016/3230109

Kanagaraj, J., Panda, R. C., & Senthilvelan, T. (2016). Green remediation of sulfide in oxidative dehairing of skin and correlation by mathematical model: An eco-friendly approach. *Process Safety and Environmental Protection, 100*, 36-48. https://doi.org/10.1016/j.psep.2015.12.005

Król-Morkisz, K., & Pielichowska, K. (2019). Thermal decomposition of polymer nanocomposites with functionalized nanoparticles. In K. Pielichowski, & T. M. Majka (Eds.), *Polymer composites with functionalized nanoparticles* (pp. 405-435). Elsevier. https://doi.org/10.1016/B978-0-12-814064-2.00013-5

Kumari, A., Mahapatra, P., Garlapati, V. K., & Banerjee, R. (2009). Enzymatic transesterification of jatropha oil. *Biotechnology for Biofuels, 2*, 1. https://doi.org/10.1186/1754-6834-2-1

Lopez, M. J., & Mohiuddin, S. S. (2020). *Biochemistry, essential amino acids*. StatPearls Publishing.

Mohamed, R. R., Fahim, M. E., & Soliman, S. M. A. (2022). Development of hydrogel based on carboxymethyl cellulose/poly(4-vinylpyridine) for controlled releasing of fertilizers. *BMC Chemistry, 16*, 522. https://doi.org/10.1186/s13065-022-00846-6

Muralidharan, V., Palanivel, S., & Balaraman, M. (2022). Turning problem into possibility: A comprehensive review on leather solid waste intra-valorization attempts for leather processing. *Journal of Cleaner Production, 367*, 133021. https://doi.org/10.1016/j.jclepro.2022.133021

Mutunga, M. F., Wanyonyi, W. C., & Ongera, G. (2020). Utilization of macadamia seed husks as a low-cost sorbent for removing cationic dye (basic blue 3 dye) from aqueous solution. *Environmental Chemistry and Ecotoxicology, 2*, 194-200. https://doi.org/10.1016/j.enceco.2020.09.005

Never, Z., Cecilia, C., Suleimain, M., & Madundo, M. (2019). Dehairing of animal hides and skins by alkaline proteases of aspergillus oryzae for efficient processing to leather products in Tanzania. *African Journal of Biotechnology, 18*(20), 426-434. https://doi.org/10.5897/AJB2019.16752

Nyakundi, J. O., Ombui, J. N., Mulaa, F. J., & Wanyonyi, W. C. (2021). Evaluation of the bacillus cereus strain 1-p protease for the unhairing of goatskins during leather production. *Textile & Leather Review, 4*(1), 38-54. https://doi.org/10.31881/TLR.2020.18

Osawa, W. O, Sahoo, P. K. Onyari J. M., & Mulaa F. J. (2015). Experimental investigation on performance, emission and combustion characteristics of croton megalocarpus of biodiesel blends in a direct injection diesel engine. International *Journal of Science and International Technology, 4*(1), 26-33.

Osawa, W. O, Sahoo, P. K. Onyari J. M., & Mulaa F. J. (2016). Effects of antioxidants on oxidation and storage stability of croton megalocarpus biodiesel. *International Journal of Energy and Environmental Engineering, 7*, 85-91. https://doi.org/10.1007/s40095-015-0191-z

Prayitno, P., Rahmawati, D., & Griyanitasari, G. (2017). Sheep wool-derived hydrolyzed keratin from tannery waste of the tanning industry using perhydrol. *Majalah Kulit Karet dan Plastik* [*Rubber and Plastic Leather Magazine*], *33*(2), 73-78. https://doi.org/10.20543/mkkp.v33i2.3336

Rajabinejad, H., Zoccola, M., Patrucco, A., Montarsolo, A., Rovero, G., & Tonin, C. (2018). Physicochemical properties of keratin extracted from wool by various methods. *Textile Research Journal, 88*(21), 2415-2424. https://doi.org/10.1177/0040517517723028

Saranya, R., Prasanna, R., Jayapriya, J., Aravindhan, R., & Tamil Selvi, A. (2016). Value addition of fish waste in the leather industry for dehairing. *Journal of Cleaner Production, 118*, 179-186. https://doi.org/10.1016/j.jclepro.2015.12.103

Senthilvelan, T., Kanagaraj, J., & Mandal, A. B. (2012). Application of enzymes for dehairing of skins: Cleaner leather processing. *Clean Technologies and Environmental Policy, 14*, 889-897. https://doi.org/10.1007/s10098-012-0458-5

Sivasubramanian, S., Manohar, B. M., & Puvanakrishnan, R. (2008). Mechanism of enzymatic dehairing of skins using a bacterial alkaline protease. *Chemosphere, 70*, 1025-1034. https://doi.org/10.1016/j.chemosphere.2007.07.084

Teixeira, W. F., Fagan, E. B., Soares, L. H., Soares, J. N., Reichardt, K., & Neto, D. D. (2018). Seed and foliar application of amino acids improve variables of nitrogen metabolism and productivity in soybean crop. *Frontiers in Plant Science, 9*. https://doi.org/10.3389/fpls.2018.00396

Tsuda, Y., & Nomura, Y. (2014). Properties of alkaline-hydrolyzed waterfowl feather keratin. *Animal Science Journal, 85*(2), 180-185. https://doi.org/10.1111/asj.12093

Van Der Ham, A. G. J. M. (2021). Process for dehairing and liming of hides, skins or pelts. *WO2021101381A1*. https://patents.google.com/patent/WO2021101381A1/en

Wanyonyi, W. C., & Mulaa, F. J. (2019). *Alkaliphilic enzymes and their application in novel leather processing technology for next-generation tanneries*. Springer. https://doi.org/10.1007/10_2019_95

Wanyonyi, W. C., Onyari, J. M., Shiundu, P. M., & Mulaa, F. J. (2016). New eco-friendly and clean method of processing hides and fish skins into leather using alkaline protease enzyme. *WO2016/159394A8*. https://patents.google.com/patent/WO2016159394A1/en

Wanyonyi, W. C., Onyari, J. M., Shiundu, P. M., & Mulaa, F. J. (2014). Enzymatic decolorization of malachite green dye by a newly isolated bacillus cereus strain wwcp1. *IOSR Journal of Environmental Science, Toxicology and Food Technology, 8*(12), 58-64. https://doi.org/10.9790/2402-081235864

Yorgancioglu, A., Başaran, B., & Sancakli, A. (2020). Value addition to leather industry wastes and by-products: Hydrolyzed collagen and collagen peptides. In A. Korlu (Ed.), *Waste in textile and leather sectors*. IntechOpen. https://doi.org/10.5772/intechopen.92699

Zhou, C., Qin, H., Chen, X., Zhang, Y., Xue, Y., & Ma, Y. (2018). A novel alkaline protease from alkaliphilic idiomarina sp. C9-1 with potential application for eco-friendly enzymatic dehairing in the leather industry. *Scientific Reports, 8*, 16467. https://doi.org/10.1038/s41598-018-34416-5