Application of Acid Protease for Eco-friendly Pre-treatment of Goat Skin to Improve Antimicrobial Finish Using Herbal Natural Extracts

by
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Abstract

Due to its moisture retention capacity and huge surface area, leather is highly prone to microbial proliferation and biodeterioration; hence, leather products desired have an antimicrobial finish. In this study, acid protease enzyme pre-treatment of goat skin was utilized as an eco-friendly substitute for conventional wet-chemical processing. The treatment can impart the desired surface properties to improve the antimicrobial finish with natural extracts obtained from leaves of *Azadirachta indica* (Neem Tree), *Ocimum sanctum* (Holy Basil, Tulsi), and *Camellia sinensis* (Green Tea). The procedure was optimized for different process parameters, including enzyme concentration, pH, material to liquor ratio (MLR), treatment time, and temperature. The effect of the treatment on bulk and surface properties of the skin was characterized by weight loss analysis, X-Ray Diffraction (XRD), Thermal Gravimetric Analysis (TGA), and Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR), X-Ray Photoelectron Spectroscopy (XPS), Water contact angle measurement, Scanning Electron Microscopy (SEM) respectively. The effect of the enzymatic treatment on organoleptic properties and the mechanical strength of the skin was also studied. The enzymatic treatment resulted in weight loss, and removal of non-collagen components, thus opening the fibrous collagen matrix of the skin. Hence, the skin treated with acid protease enzyme provides better affinity and accessibility for phytoactive compounds from the natural extracts and better attachment by electrostatic attachment due to an increase in surface functional groups after the enzymatic treatment compared to untreated skin. The effectiveness of the antimicrobial finish was measured as a zone of inhibition and with a modified Hohenstein test against test microorganisms *E. coli and S. aureus. Azadirachta indica* (Neem Tree) extract showed the highest inhibitory activity (97%) against *E. coli*, while the *Ocimum sanctum* (Holy Basil, Tulsi) extract exhibited the highest inhibitory activity (95%) against *S. aureus*.

Introduction

Leather/leather products have become a valued commodity in the global market. Also, the leather/leather product manufacturing industries have emerged as a prime economic sector in developing countries.[1] Despite having a prominent role in economic development worldwide, the leather production industries continually face challenges. Due to environmental pollution, the leather/leather product manufacturing industries are under strict criticism by societies and pollution control authorities.[2] Leather produced from flayed animal skins by a series of operations/treatments briefly classified under beam hose/pre-tanning, tanning, and post-tanning and finishing. In the conventional process of leather manufacturing, a substantial quantity of water and various chemicals are required at each stage of sequential operations.

Additionally, a massive quantity of chemicals is required to impart/enhance the desirable qualities of the final product. Thus, conventional leather production processes expel massive amounts of pollutants, including hazardous chemicals, into the environment. The conventional wet-chemical treatment generates wastewater that requires extensive treatment and is a source of environmental pollution even after treatment and disgorges toxic gases like ammonia and hydrogen sulfide.[2–4]

As a result of the emergence of awareness in society, strict legislation, and global concern about the environmental impacts caused by leather industries, the industries are under constant pressure to opt for alternatives, cleaner and greener processing which can limit the usage of hazardous/other chemicals in processing. The utilization of enzymes in various pre-tanning operations and incorporation of enzymes in post-tanning operations to impart required properties to the processed leather is gaining considerable importance as an alternative eco-friendly approach.[4–6] Various enzymes such as collagenase, keratinase, protease, and lipase are being explored and utilized for leather processing.[5] These enzymes are reported to soften the collagen fiber matrix, augment the characteristics of the leather, and significantly can lower environmental pollution by reducing the use of hazardous and polluting chemicals. Enzyme-based skin processing has emerged as a promising technology for leather production by incorporating enzymes at various stages in leather processing.[5] However, for successful applications of enzymes in leather processing, stringent control of process parameters is obligatory to acquire substantial results,[2–4,7] and therefore researchers are working to gain a critical understanding of the application of enzymatic treatment in leather processing.[8]

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Microbial deterioration of skin/leather is another critical aspect affecting its aesthetic properties, life expectancy, and utilization of leather products.[9] Skin/leather, made up of collagen protein, is highly susceptible to the growth of microorganisms due to its large surface area and moisture retention capacity.[10,11] Consumer awareness toward personal hygiene and health risk associated with dreadful microorganisms and decrease in life expectancy due to microbial deterioration also seeks to impart antimicrobial properties/finish to leather/leather products. For imparting antimicrobial finish, various synthetic organic chemical agents are under use; however, many of them are now banned due to ecological toxicity and growing environmental concern.[9,12] Recently, the application of natural antimicrobial agents to impart antimicrobial properties to leather/leather products has attracted significant attention from researchers worldwide as they are environment-friendly, skin-friendly, safe, non-toxic, and exhibit broad-spectrum activity.[13,14]

The finishing of skin/leather is challenging because of variations in the three-dimensional matrix of collagen fiber weave in skin/leather. Particularly, absorption of antimicrobial agents by skin/leather varied because of differences in affinity and accessibility and the presence of non-collagenous materials in the skin. The conventional wet-chemical pre-treatment to impart desired finish is not environmentally friendly; also, the bulk properties of skin/leather may be affected during the conventional wet-chemical pre-treatment. Thus, there is a quest for appropriate eco-friendly methods to modify the collagen weave matrix of the skin and remove non-collagenous material without affecting its bulk properties.[15] The use of acid protease enzyme as an alternative to the wet-chemical treatment is investigated in this study. Enzymatic treatment of skin/leather can remove/reduces interfibrillar cementing substances like sulfated glucosamine glycans, uronic acid, hexosamines, and other glycoproteins from skin/leather[16] and results in fiber opening, the opening of collagen bundle weave[17] and ultimately results in better absorption and affinity for a finishing agent.[2,18,19] Among the enzymes used for skin/leather processing, the protease is widely investigated for its isolation, screening, production optimization, purification, characterization, and applications,[20] particularly for substitution of chemical dehairing,[8,15,20] waste treatment.[21] Apart from this, the application of collagenolytic protease (collagenase) and acid protease as eco-friendly pre-treatment was reported for improved dyeing of leather due to better absorption and diffusion of dyes in the leather matrix due to well-opened collagen fiber matrix.[22,23]

In this study, acid protease enzyme pre-treatment of goat skin was investigated as an environment-friendly alternative to conventional wet-chemical processing to improve antimicrobial finish with natural extracts. The pre-tanning stage goat skin was treated with acid protease at variable process parameters for optimization of enzymatic treatment, and the enzymatic treatment-induced changes in bulk and surface properties were studied using various characterization techniques. Subsequently, the skin was treated with the natural extracts obtained from leaves of *Ocimum sanctum* (Tulsi, Holy Basil), *Azadirachta indica* (Neem Tree), and *Camellia sinensis* (Green tea) to impart antimicrobial activity, which is tested against test microorganisms *E. coli and S. aureus* with the zone of inhibition and modified Hohenstein test.

Experimental Work

2.1 Material:

Full-grain goat skin with all pre-tanning treatments was used in this study for enzymatic treatment and subsequent antimicrobial finishing. The goat skin was purchased from a tannery in Agra city, Uttar Pradesh, India. The skin has an average thickness of 1mm, cut into pieces of appropriate size for experimental study. The acid protease enzyme (Activity~ 4000 units/mg, Product code RM6186, Source: *Aspergillus spp.*), citric acid, and agar powder used in this study were purchased from HiMedia Laboratories, Mumbai, India. Luria Broth used in this study was purchased from SRL chemicals, India. The enzyme 'acid protease' used in the study is a commercial product of Himedia Company. The company provided the enzyme activity and stability data; the enzyme's shelf life is three years upon storage in the recommended storage condition. After preliminary experiments, the optimum conditions were determined to be 60 minutes, pH 4.5, MLR 1:20, temperature 50°C, and enzyme concentration 1%w/v. Leaves of *Azadirachta indica* (Neem Tree) and *Ocimum sanctum* (Holy Basil, Tulsi) were collected from the campus garden of Manipal University Jaipur, Rajasthan. Leaves of *Camellia sinensis* (Green Tea) were purchased from a local vendor. The leaves were washed with distilled water to remove dust and impurities, then air dried, converted to powder using a domestic mixture grinder, sieved (U.S. Standard Screen Size No. 35) and subsequently used to obtain the antimicrobial extract.

2.2 Enzymatic Treatment of Goat Skin Samples with Acid Protease:

The goat skin was cut into pieces of 5cm x 5cm and provided enzymatic treatment with the 1% w/v acid protease enzyme solution prepared in phosphate buffer at pH of 4.5,[22] material to liquor ratio (MLR) of 1:20, at 50°C for 60 minutes. The effect of time on the acid protease treatment of goat skin was studied by varying treatment times from 40 min to 70 min. Further, the effect of the acid protease enzyme concentration was studied by conducting experiments with above mention condition with enzyme concentration in the range of 0.5 to 2% w/v prepared in phosphate buffer. The material-to-liquor ratio varied in the range of 1:10 to 1:40. The temperature of the enzymatic treatment was optimized by providing treatment at different temperatures of 30°C to 60°C with other similar experimental conditions mentioned above. For the effect of pH on acid protease enzyme treatment of the goat skin, the pH of enzyme solution was set in the range of 4 to 5.5 using phosphate buffer and goat skin treated with enzyme concentration of 1% w/v, MLR of 1:20 at 50°C for 60 minutes. In all the experiments, enzymatic treatment was stopped by removing goat skin pieces from the acid protease solution and dipping them in hot water at 70°C for 5 seconds. After that, enzyme-treated skin pieces were washed thoroughly with doubled distilled water and dry in ambient conditions, followed by drying in a hot air

oven at 70°C for 24 hours to remove moisture. All the experiments were performed three times, and from obtained results, the average value and standard deviation were calculated for quantitative analysis. For comparison, control samples were prepared for all the above experimental conditions by dipping the goat skin pieces in a phosphate buffer solution without the acid protease enzyme.

2.3 Characterization:

The goat skin's surface and bulk properties, the effect of the enzymatic treatment on the surface, and the bulk properties of the goat skin were studied using various characterization techniques. Changes in the bulk properties of the goat skin due to the enzymatic treatment were studied by weight loss % measurement, analysis of physical properties by TG and DTG analysis, XRD analysis, SEM, analysis of Organoleptic properties, and analysis of mechanical properties such as tensile strength, % elongation at break, and tear strength. Effect of the enzyme treatment on non-collagen components of the goat skin studied by estimation before and after. Changes in the surface chemical composition of the goat skin by the enzymatic treatment studied by ATR-FTIR and XPS analysis of the grain surface of the goat skin and resultant improvement in wettability studied by water contact angle measurement at the grain surface. A detailed description of the characterization experiments is provided in the supplementary data.

3 Preparation of antimicrobial extract and antimicrobial finishing of Goat skins:

Natural antimicrobial extracts were prepared from the dry powder of leaves of *Azadirachta indica* (Neem Tree), *Ocimum sanctum* (Holy Basil, Tulsi), and *Camellia sinensis* (Green Tea) and phytochemical analysis of the prepared extracts was carried out. Identification of active constituents of the extracts was carried out using UV-visible spectroscopy and High-performance Liquid Chromatography (HPLC). The natural extracts were used for the antimicrobial finishing of the goat skin using citric acid as a binding agent. The antimicrobial finish and effect of the enzymatic treatment on the antimicrobial finish were studied using the Agar diffusion test (AATCC Test 90-2011; Agar plate method) and modified Hohenstein test (JIS L 1902). *Staphylococcus aureus* (MTCC9542) (Gram-positive organism) and *Escherichia coli* (ATCC 25922) (Gram-negative organism) were utilized as test microorganisms. The details of the experimental condition for the preparation of antimicrobial extracts, characterization, and identification of active constituents and obtained results, along with experimental details of antimicrobial finish and testing of antimicrobial finish, are provided as supplementary data.

4 Results and Discussion

4.1 Weight loss percentage:

The weight loss observed after acid protease treatment of goat skin samples may be due to breaking proteins into amino acids. In this study, the measurement of weight loss percentage was utilized to study the effect of different treatment parameters on the performance of the acid protease enzyme.

The effect of treatment time on acid protease resultant weight loss is presented in Figure 1(a). From Figure 1 a, it can be inferred

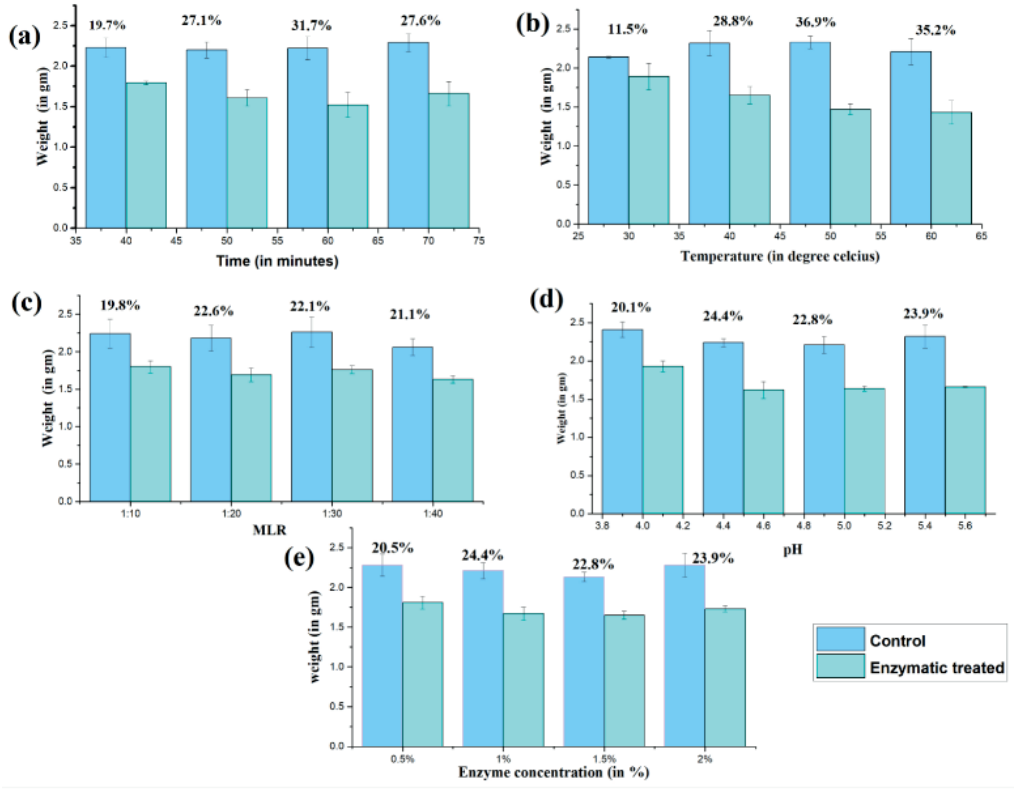

Figure 1. Weight of control and enzymatic treated goat skin for different treatment variables
a) Treatment time **b)** Temperature **c)** Material to liquor ratio **d)** pH **e)** Concentration of the enzyme.

that the maximum weight loss (31.7%) occurred for 60 minutes of treatment time. The acid protease treatment of goat skin samples beyond 60 minutes does not increase weight loss; the substrate may become one of the limiting factors by becoming depleted over time, resulting in no further interaction between the enzyme and substrate. These factors contribute to the constant weight of the goat skin beyond 60 minutes.[24–27] Figure 1(b) presents the effect of temperature on acid protease treatment resulting in the weight loss percentage of goat skin. Weight loss percentage increase with the increase in treatment temperature from 30°C to 50°C; there is a rise in weight loss percentage from 11.5% at 30°C to a maximum of 36.9% at 50°C because the increase in treatment temperature increases inter and intramolecular kinetic energy, resulting in more collision of enzymes and substrate, thereby enhancing the reaction rate. When the treatment temperature increased beyond 50°C, a slight decrease in weight loss percentage was observed. This decrease may be caused by the high internal energy of enzyme molecules that result from vibration, which stresses the amino acid bonds at the reactive site and changes the enzyme protein's secondary, tertiary, and quaternary structures. These weak connections are broken by high temperatures, which eventually causes the enzyme's activity to decline.[24,28] Weight loss % increased when the material to liquor ratio increased from 1:10 to1:20, but as the ratio increased further, the weight loss percentage decreased. This increase is due to the enzyme's lower diffusion rate when the MLR (Material to liquid ratio) increases (Figure 1(c)).[24,29] As presented in Figure 1(d), the optimum performance of the enzyme treatment was observed at pH 4.5, providing a maximum of 27.8% weight loss. At a pH other than 4.5, a decrease in the enzyme performance is observed because as the pH deviates from its optimum range, the amino acid composition of the reactive site changes so that the substrate can no longer react with the enzyme.[28] The impact of enzyme concentration on weight loss percentage was investigated at different concentrations ranging from 0.5% to 2% (w/v). As shown in Figure 1 (e), the weight loss percentage increased with an increase in the enzyme concentration from 0.5 % to 1%. Maximum 24.4% weight loss occurred at 1% w/v enzyme concentration. An increase in the enzyme concentration further than 1% did not increase weight loss, which might be due to the unavailability of substrate for the increased enzyme concentration.[24,28]

4.2 ATR-FTIR analysis:

ATR-FTIR spectrum of the upper surface of the goat skin obtained before and after the enzymatic treatment (Enzyme concentration: 1% w/v, pH: 4.5, MLR: 1:20, Temperature: 50°C, Treatment time: 60 minutes) to study changes in the chemical composition of the skin sample due to the enzyme treatment. Figure 2 shows the ATR-FTIR spectrum of untreated and enzymatic-treated goat skin. The skins are mainly composed of collagen, a fibrous protein with a triple helix structure arranged in microfibril, macro-fibril, and fibril bundles that are tightly woven to form a three-dimensional matrix of skin/leather. The acquired spectra matched the collagen IR spectrum, and the corresponding peaks were identified.

In the IR spectrum of proteins, the peptide group, a structural repeat unit of protein, provides nine characteristic peaks known as amide A, amide B, and amide I to VII. In the obtained IR spectrum, the most intense absorption peak in the 1616 cm^{-1} is identified as Amide I, which primarily gives rise due to the stretching vibration of C=O and, to an extent, C-N groups. The peak in the range of 1510 cm^{-1} and 1580 cm^{-1} is identified as Amide-II, which gives rise due to N-H bending coupled with C-N stretching vibration. Amide-I and amide II are complex peaks modulated by the protein's secondary and tertiary structure; hence, collagen's backbone configuration and bonding determine the exact peak position of Amide I and Amide II 1.[30] Peaks obtained at 1321 cm^{-1}, 1232 cm^{-1}, and 1196 cm^{-1} are due to the C-N stretching and N-H bonding vibration from amide linkage as well as wagging vibration of CH_2 group in glycine backbone and prolines and assigned as Amide-III.[31] The peak appeared at 1450 cm^{-1} arising from typical amide vibration assigned to C-N stretch and N-H wagging. The peak at 1196 cm^{-1} and 1111 cm^{-1} is assigned to C-O-C, and the peak at 1031 cm^{-1} is assigned to C-O stretching vibration.[1] The absorption peaks at 2852 cm^{-1} and 2921 cm^{-1} are assigned to the aliphatic C-H, CH_2, CH_3 stretching of the aliphatic side chain of amino acid. The peak at 3075 cm^{-1} and 3300 cm^{-1} overlapped with a broad peak due to the OH group identified as

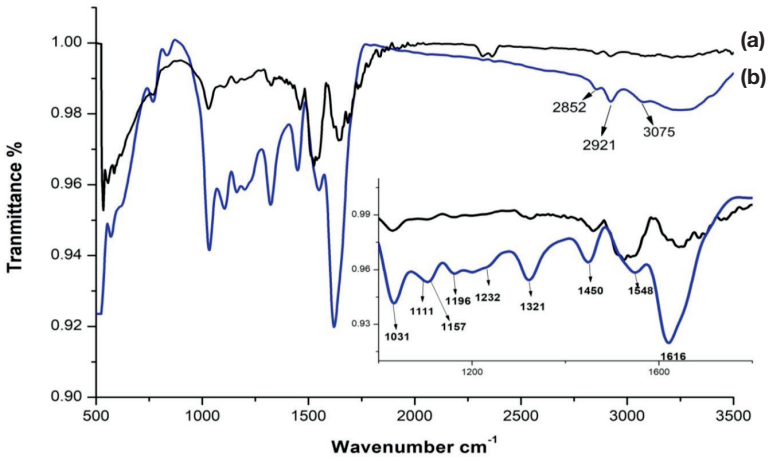

Figure 2. FTIR analysis of a) untreated b) enzymatically treated goat skin

Amide B and Amide A, respectively. These peaks are derived from a fermi resonance between the first inference of amide II and N-H stretching vibration.[1]

As seen in Figure 2, no changes in the overall spectrum of skin treated with the enzymatic treatment were observed compared to the IR spectrum of the untreated skin. Enzymatic treatments profoundly affect the peak positions and result in the development of some new peaks without affecting the overall spectrum. The enzymatic treatment increased the intensity of peaks in general; the intensity of peaks within 1196-1111cm^{-1} increased in the spectrum of the enzyme-treated skin.[1,32] These indicate enzymatically induced changes in the chemical composition of the grain surface of the skin. The enzymatic treatment did not result in changes in the position of major amide peaks, but after the enzymatic treatment, the intensity of Amide III and Amide II increased and the peaks broadened to some degree. In the case of the enzyme-treated skin, the peak position of amide I in the spectrum shifted a little to 1616cm^{-1}, along with a shape change and significant increase in intensity. As amide bands (particularly amide I) are comprised of various bands dependent on secondary conformations of collagen, the changes noted in the IR spectrum of the enzymatically treated skin imply a slight modification of collagen due to the enzyme treatment and removal of denatured collagen. The difference between the peak position of Amide I and Amide II indicates the presence of denatured collagen if the difference is higher than 100cm^{-1}. However, in both, the spectrum difference between the peak position is less than 100cm^{-1} and lower in the case of enzymatically treated skin compared to untreated skin. So it can be concluded that the enzymatic treatment had not affected the triple helix conformation of collagen but removed the denatured protein from the skin.[1,30,33] Apart from this, the enzyme-treated skin decreased intensity, and a shift in the position of amide B was observed. The alkyl C–H stretching bands at 2921cm^{-1} and 2852cm^{-1} remain unaffected; the Amide A and Amide B peaks became intense.

4.3 XPS analysis:

XPS analysis of grain surface of untreated and enzyme-treated skin was carried out to study changes in chemical composition due to the enzymatic treatment (Enzyme concentration: 1% w/v, pH: 4.5, MLR: 1:20, Temperature: 50°C, Treatment time: 60 minutes). Figure 3 presents the XPS survey scan for the untreated and the

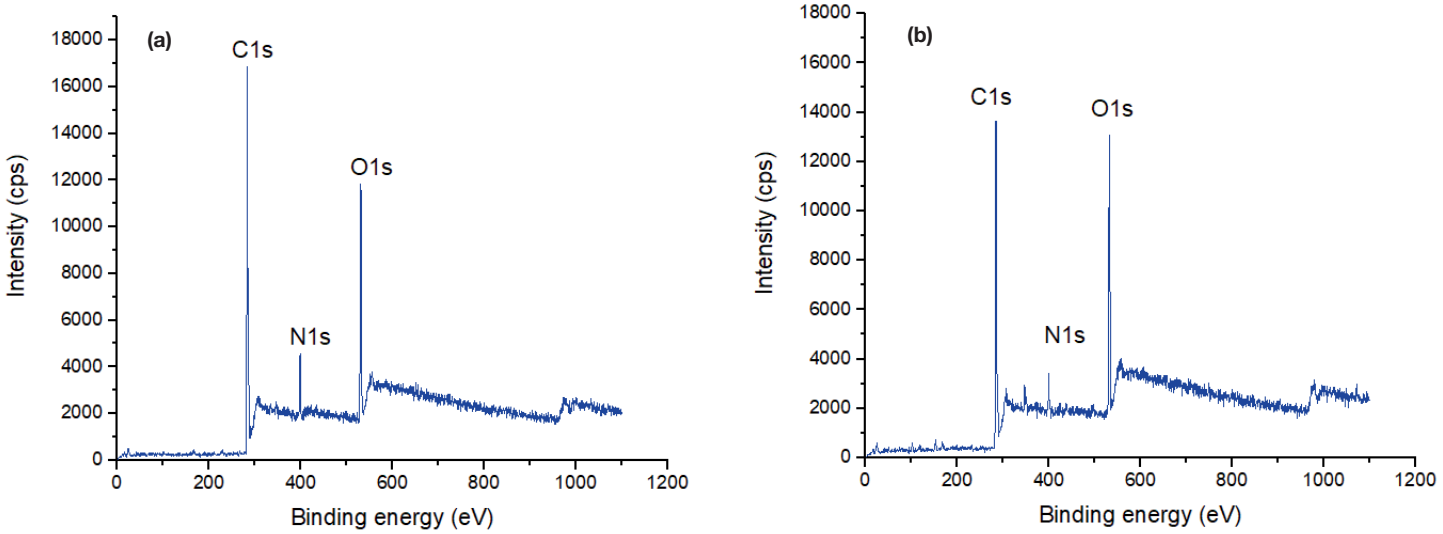

Figure 3. XPS survey scan spectra of **a)** untreated and **b)** enzymatically treated goat skin

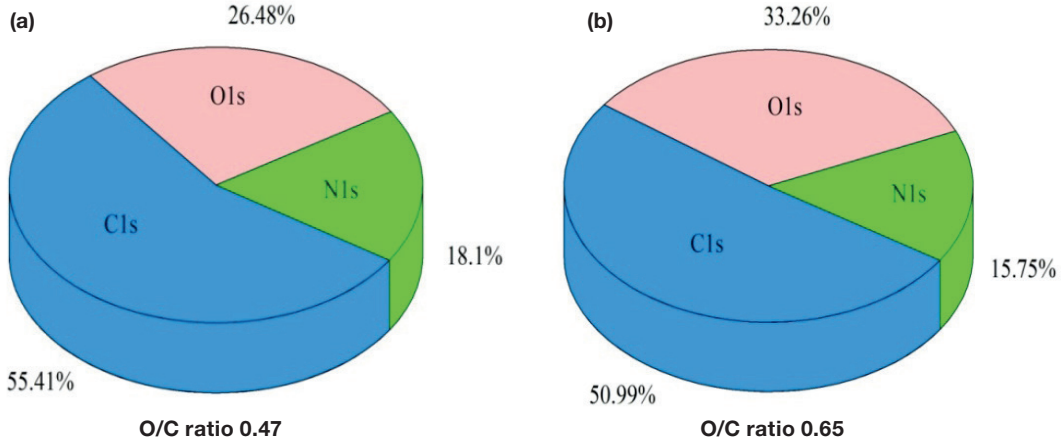

Figure 4. Elemental composition of **a)** untreated **b)** enzymatically treated goat skin

enzyme-treated goat skin. As revealed in Figures 3 and 4, the elemental composition of the grain surface of the goat skin changed significantly after the enzyme treatment. XPS survey analysis revealed that the grain surface of the enzymatically treated goat skin surface has higher oxygen content than the grain surface of untreated goat skins, which was further quantified from the high-resolution survey. The O/C atomic ratio of untreated goat skin grain surface was found to be 0.477, which increased to 0.65 after treatment with the acid protease enzyme. The enzyme treatment increased oxygen concentration from 26.8% for untread skin to 33.2%, with an increase of 6.78% relative to untreated goat skin. This considerable upsurge in the atomic O/C ratio shows that new oxygen-containing groups are being exposed to the grain surface after the enzymatic treatment. The enzymatic treatment decreased nitrogen concentration from 18.1% for untreated skin to 15.75% for the enzyme-treated skin, and carbon content also changed. These overall transitions in surface chemistry can result from breaking peptide bonds and loss of amino acids due to the acid protease treatment. The enzyme treatment can lower the carbon content by forming certain volatile products and also help to expose the group containing oxygen.[2,4]

4.4 TG and DTG analysis:

The impact of the enzyme treatment on thermal stability and thermal degradation of the goat skin was studied by TG and DTG analysis, and the acquired TG and DTG thermograms are shown in Figure 5. As revealed in Figure 5, non-isothermal degradation of goat skin occurred in three successive stages. In the initial stage, both untreated and the enzyme-treated (Enzyme concentration: 1% w/v, pH: 4.5, MLR: 1:20, Temperature: 50°C, Treatment time: 60 minutes) skin showed similar and smooth weight loss (10%) profiles, which attributed to the loss of bound and unbound water. During this stage, moisture present in untreated and the enzyme-treated skin sample entirely lost; this stage was endothermic.[34,35] The successive stages were exothermic, involving thermo-oxidation and decomposition of the goat skin samples. The skin samples' thermic oxidation and decomposition started at 320°C and continued up to 800°C. Untreated goat skin showed a melting phase with a sharp peak at 336°C, while the enzyme-treated skin sample showed a melting phase with a sharp peak at 369°C. The results indicated that the enzyme-treated goat skin exhibited higher thermal stability and sharped weight loss than those observed for untreated skin samples 35. The obtained TGA data were converted to a DTG curve for better comprehension. As seen in Figure 5, the first peak in the DTG curve corresponds to the loss of water content from the samples, and the second peak is related to denaturing of the goat skin protein structure due to the decomposition of the peptide bonds and thermal degradation subsequently. Sharper and higher amplitude peaks obtained for the enzyme-treated skin compared to untreated skin indicate that structural alternation occurred due to the enzymatic treatment, further characterized by XRD analysis.[33]

4.5 XRD analysis:

XRD analysis was conducted to study the effects of the enzymatic treatment on goat skin's structural properties and crystalline structure. Figure 6 shows the XRD pattern of untreated goat skin and the enzyme-treated (Enzyme concentration: 1% w/v, pH: 4.5, MLR: 1:20, Temperature: 50°C, Treatment time: 60 minutes) goat skin. As seen from Figure 6, for both untreated and enzyme-treated goat skin, one high amplitude peak at 2θ value of about 20.8° and small peaks at 2θ value of about 7.1°, 30.1°, 40.2° were obtained; further details about peaks are given in Table I. The obtained XRD patterns indicate the amorphous nature of the material of which skin is composed. The sharp peak at a 2θ value of about 7.1° corresponds to the crystalline component of the skin. Whereas at 2θ value of about 20.8°, a broad peak due to the amorphous component of goat skin is observed.[37] The broad peak observed at 2θ value of about 20.8° assigned to reflections by the weave of collagen fibers which cause the diffuse peaks. The intensity of this peak represents the dimensional structure regularity of collagen and crosslinking degree.[38] A decrease in intensity of peak observed at 2θ value of about 20.8° indicates that compared to untreated goat skin, the enzyme-treated goat skin has less compact and tightened weaving

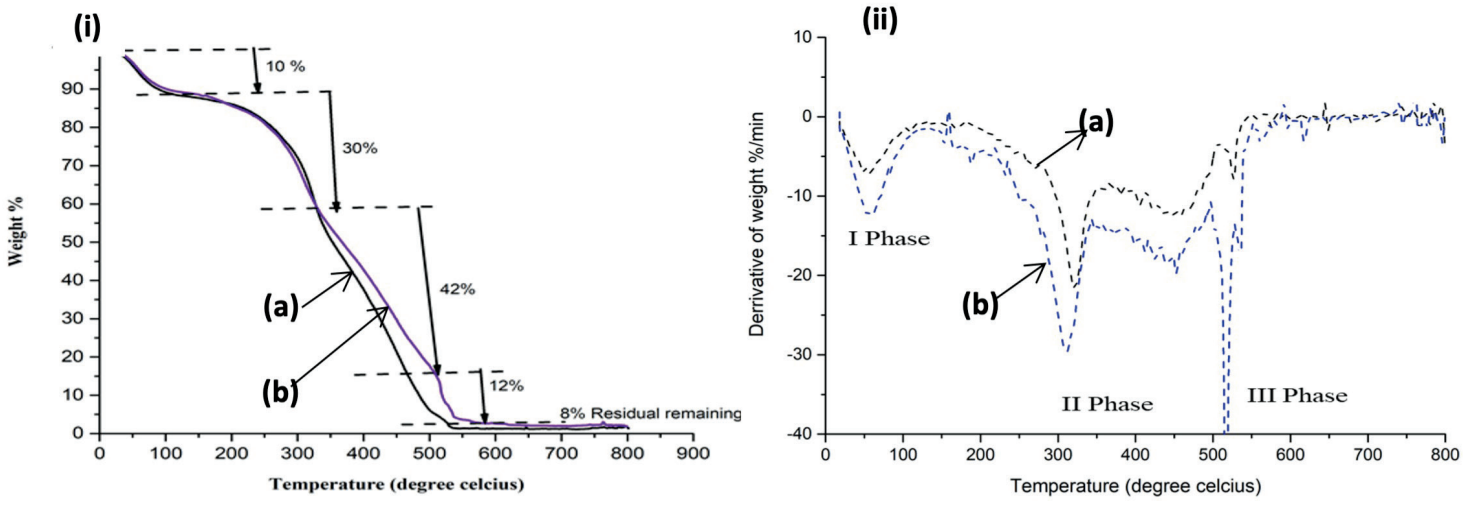

Figure 5. **i)** TGA graph **ii)** DTG curve of **a)** untreated and **b)** enzymatically treated goat skin

Table I

XRD parameter of untreated and enzymatic treated goat skin

Particular	2θ value	FWHM	d spacing (nm)	Crystallinity index
Untreated goats skin	7.1°	8.3159	12.440	73%
	20.8°	10.2353	4.267137	
	30.1°	3.6717	2.96655	
	40.2°	31.73	2.241462	
Enzymatic-treated goat skin	7.1°	8.7615	12.440	65.73%
	20.8°	10.3001	4.267137	
	30.1°	5.74588	2.96655	
	40.2°	29.303	2.241462	

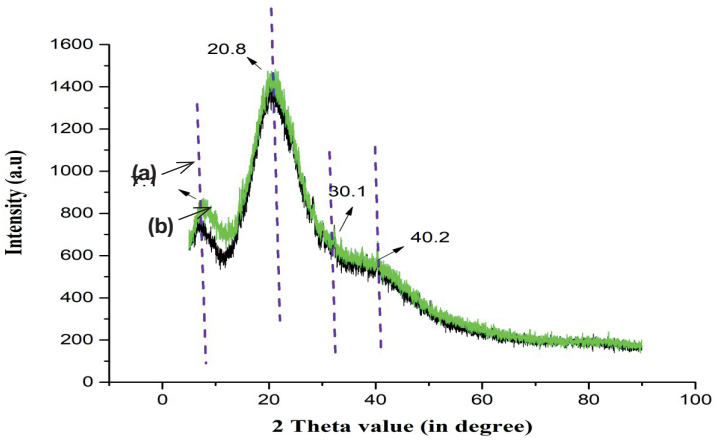

Figure 6. XRD analysis of **a)** untreated **b)** enzymatically treated goat skin

of collagen fibrils at grain surface as a consequence of the enzyme treatment.[36] The peak position of α-helix (2θ value of around 10°) and β-sheets (2θ value of around 20°) remains unaffected even after the acid protease treatment.[37,39] Thus, the enzymatic treatment with acid protease modified the structural properties of the goat skin; still, the bulk structure of the skin remained unaffected. The crystallinity index of untreated skin was about 73%, which was reduced to 65.73% in the case of enzyme-treated skin. Thus, it is worth noticing from the XRD pattern that the enzymatic treatment increased the amorphous part without affecting the bulk structure of the goat skin.[37] This amorphous portion helps increase the hydrophilicity of the skin, which ultimately affects its absorption capacity for natural antimicrobial extracts.[31,37]

4.6 SEM analysis:

Figure 7 shows the morphology of the grain surface of untreated and enzyme-treated (Enzyme concentration: 1% w/v, pH: 4.5, MLR:

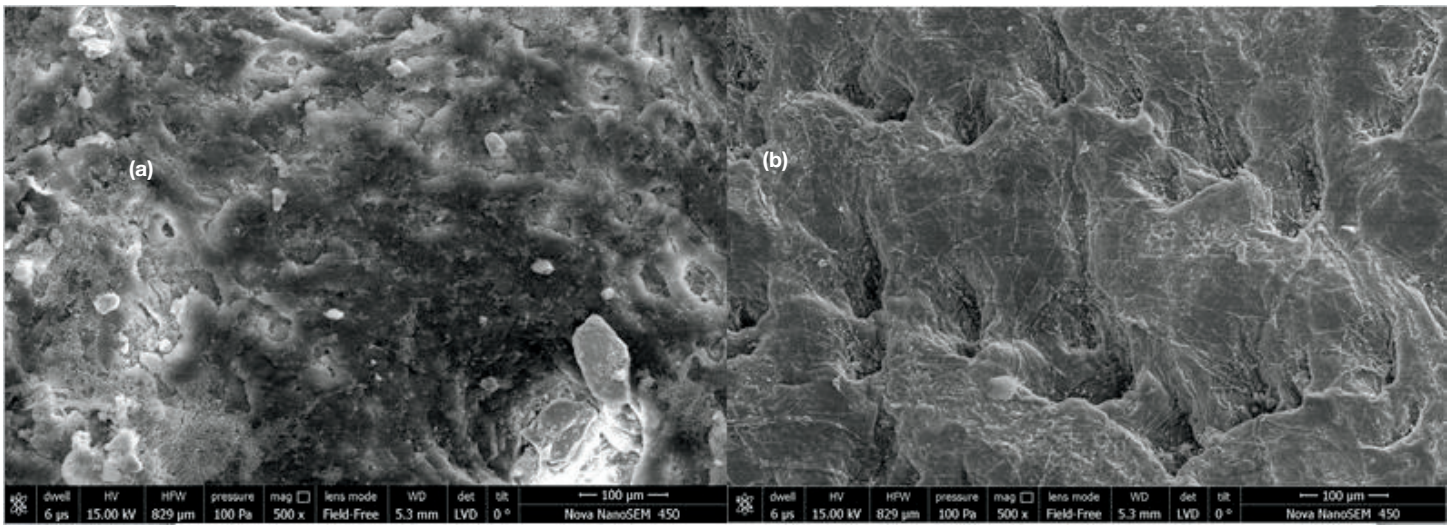

Figure 7. Morphological observations of grain surface of **a)** untreated and **b)** enzymatically treated goat skin

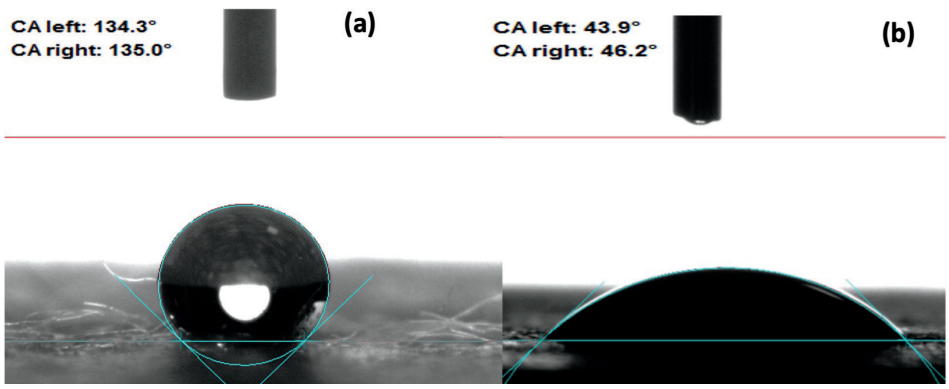

Figure 8. The water contact angle at the grain surface of **a)** untreated and **b)** enzymatically treated goat skin

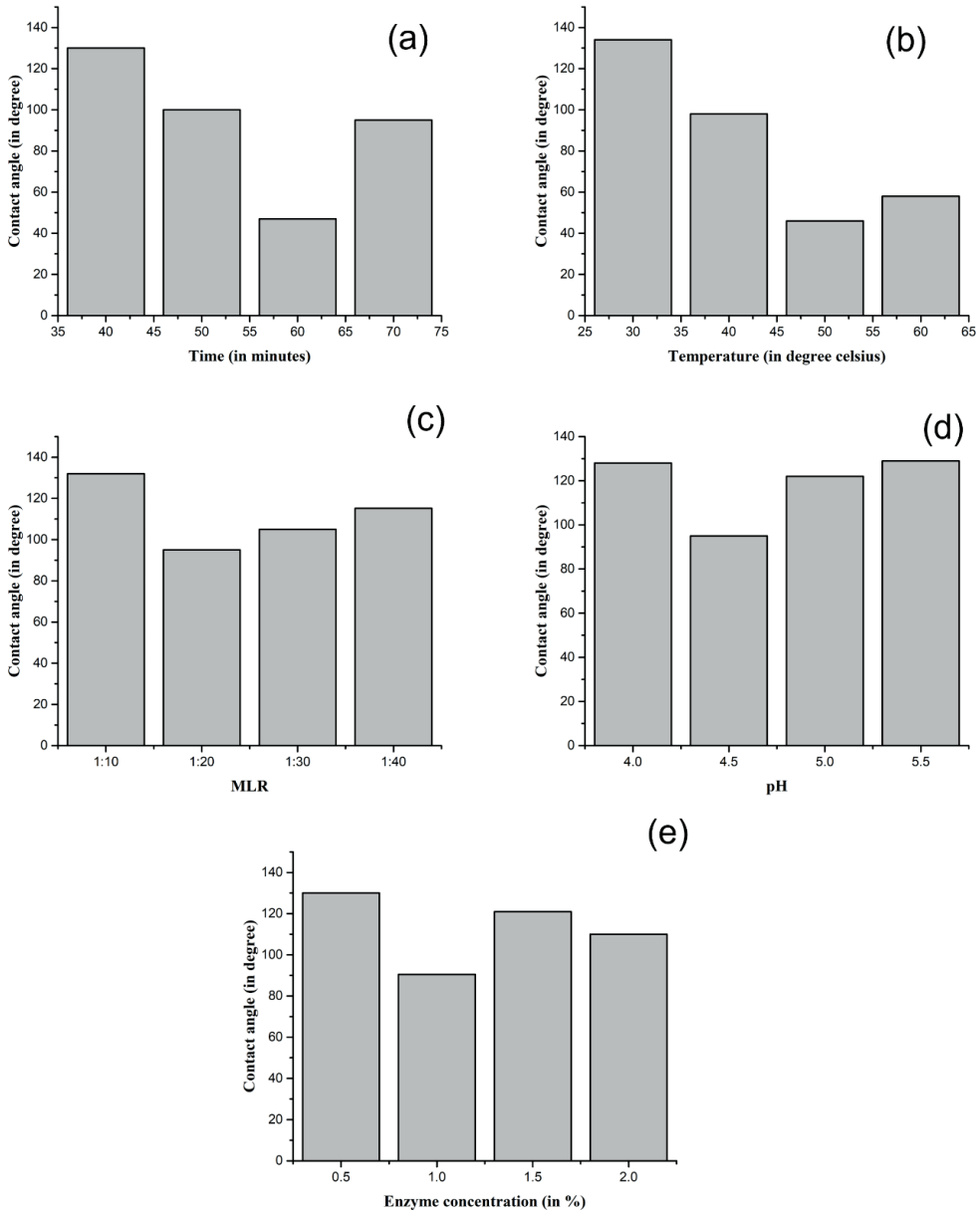

Figure 9. Changes in water contact angle at grain surface of the enzyme-treated goat skin for different treatment variables **a)** Treatment time **b)** Temperature **c)** Material to liquor ratio **d)** pH **e)** Concentration of the enzyme.

1:20, Temperature: 50°C, Treatment time: 60 minutes) goat skin. It revealed from the SEM image that the untreated skin's grain surface has a non-uniform structure with many asperities. The acid protease treatment resulted in the detachment of interfibrillar structure, opening of some fibers at the macrolevel, removal of non-collagenous substances, and modification of micropores. Thus, acid protease treatment caused the loosening of the grain surface; as revealed in Figure 7, the grain surface of goat skin became cleaner, uniform, and more homogeneous.[1]

4.7 Water contact angle measurement:

The untreated goat skin presented a hydrophobic character; when the water contact angle was measured at the grain surface of the untreated skin, the water contact angle value of 135°±.0.5 (Figure 8) obtained. After the enzymatic treatment, the wettability of the goat skin increase.

The increase in wettability/hydrophilicity of the goat skin after the enzymatic treatment and the effect of different process variables on wettability were studied using water contact angle measurement at the grain surface presented in Figure 9. For optimum enzymatic treatment conditions (Enzyme concentration: 1% w/v, pH: 4.5, MLR: 1:20, Temperature: 50°C, Treatment time: 60 minutes), the water contact angle at the grain surface of the goat skin was reduced from 135°± 0.5 to 45.6°±1 as illustrated in Figure 8. The improvement in wettability after the surface treatment was attributed to the removal of cementing substances between the collagen, impurities, modification in surface properties, and removal of nanoscale roughness at the grain surface.

4.8 Effect of the enzymatic treatment on mechanical strength and organoleptic properties:

The effect of the enzymatic treatment (Enzyme concentration: 1% w/v, pH: 4.5, MLR: 1:20, Temperature: 50°C, Treatment time: 60 minutes) was analyzed on the mechanical strength of the skin as tensile strength, elongation at break, and tear strength. The results of the mechanical strength analysis are reported in Table II. The results reveal that the enzymatically treated goat skin has slightly better mechanical properties than untreated. Due to enzymatic treatment, the surface of the goat skin becomes more hydrophilic. It was observed from SEM analysis that the enzyme-treated goat skin has well defined porous and opened fiber structure. Due to the collagen's open structure and impurities' removal, the goat skin's tensile strength has slightly increased.[2,6] The results for organoleptic properties are presented in Figure 10. In analyzing organoleptic properties by visual and hand assessment, the enzyme-treated skin was found to have comparable results with that of untreated skin. The enzyme treatment modified the skin's

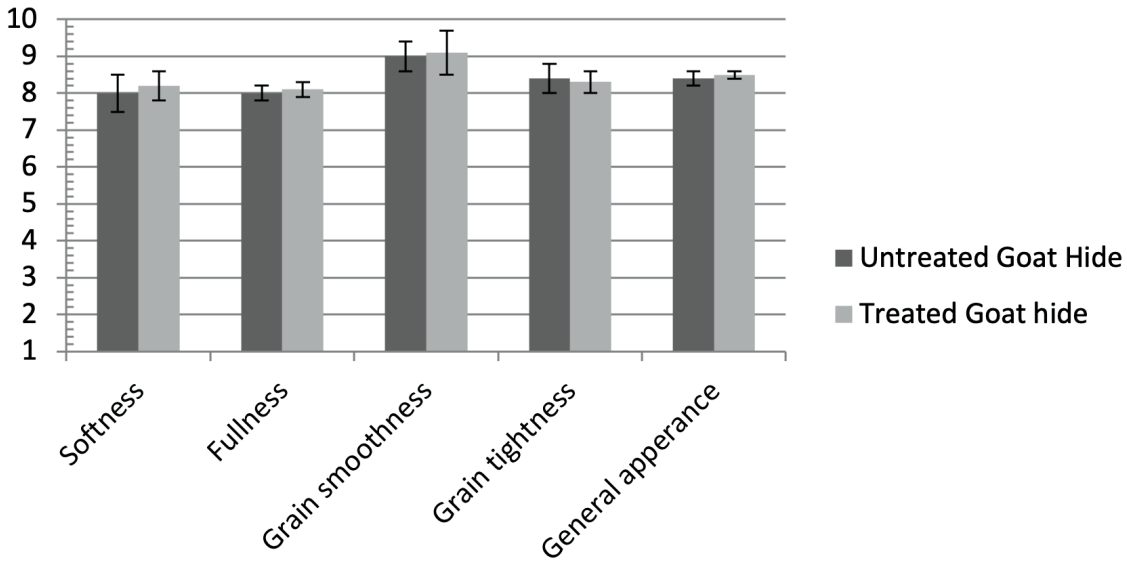

Figure 10. Assessment of organoleptic properties of untreated and enzyme-treated goat skin; the presented result is an average value of 3 determinations: Scale of 1-10, Poor-1, Best- 10

Table II

Results of mechanical strength analysis for untreated and enzyme-treated goat skin.

Experiment	Tensile strength (Kg/cm²)	% Elongation at break	Tear strength (Kg/cm)
Untreated Skin	190	78	60
Enzyme treated skin	200	81	75

Table III

Quantitative analysis of non-collagen skin components of the skin.

Non-collagen components	Control	Enzyme treated skin
Sulfated GAG (µg/g)	480	785
Uronic acid (µg/g)	2457	1000
Hexosamines (µg/g)	3485	1100

grain surface by opening the collagen matrix and removing impurities and asperities, providing better organoleptic properties such as softness, smooth grain surface, fullness, and appearance, as shown in Figure 10.[18,19]

4.9 Effect of the Enzymatic Treatment on Non-collagen Components of the Goat Skin:

Quantitative estimation of non-collagen components of untreated and enzyme-treated skin (Enzyme concentration: 1% w/v, pH: 4.5, MLR: 1:20, Temperature: 50°C, Treatment time: 60 minutes) was performed to analyze the effect of the enzymatic treatment. Reduction in interfibrillar constituents (Uronic acid, Hexosamines) was observed after the enzymatic treatment, whereas sulfated glucose amine glycan increased after the enzymatic treatment. The reduction in non-collagen components was significant in the enzymatic process due to the site-specific action of the enzyme; the results are presented in Table III. In skin/leather's triple matrix structure, collagen fiber is more densely packed due to these interfibrillar substances. These non-collagenous substances hinder diffusion and affect goat skin's adsorption/uptake of chemicals/finishing agents, which requires penetration during processing/finishing. The degree of opening of the collagen matrix of skin/leather is directly related to the extent of removal of interfibrillar substances. So, eliminating these substances is an inevitable step for producing better quality skin.[2,40]

4.10 Identification of active constituents of the natural extracts:

4.11 Antimicrobial activity:

To impart antimicrobial finish, untreated and enzyme-treated (Enzyme concentration: 1% w/v, pH: 4.5, MLR: 1:20, Temperature:

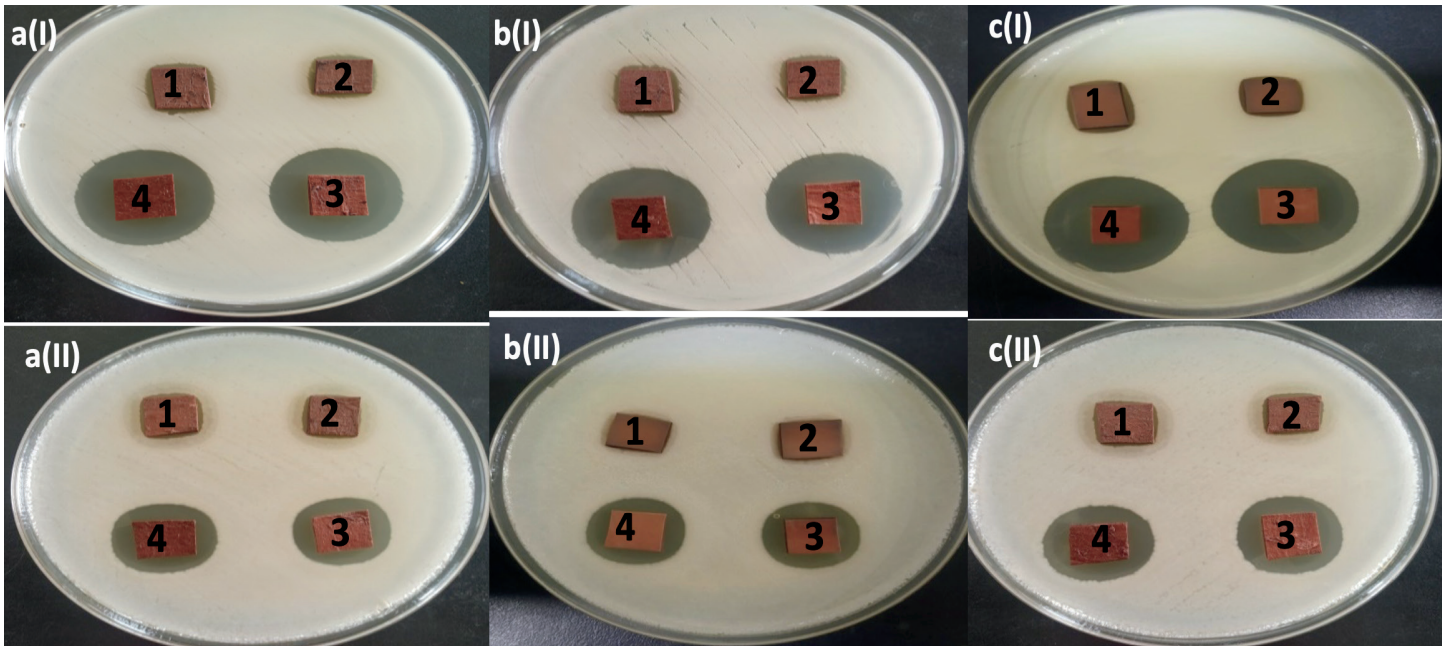

Figure 11. Zone of inhibition obtained due to antimicrobial finish with 5% w/v extract of leaves of a *Azadirachta indica* against *Escherichia coli* **a(i)** and *Staphylococcus aureus* **a(ii)**; b *Ocimum sanctum* against *Escherichia coli* **b(i)** and *Staphylococcus aureus* **b(ii)**; c *Camellia sinensis* against *Escherichia coli* **c(i)** and *Staphylococcus aureus* **c(ii)**. 1- Pieces of untreated skin, 2- Pieces of skin with the enzymatic treatment, 3- Pieces of untreated skin finished with the natural extracts, and 4- Pieces of enzyme-treated skin finished with the natural extracts, respectively.

Table IV

Zone of inhibition (cm) obtained for untreated skin finished with the natural extracts (UT+EX) and the enzyme-treated skin finished with the natural extracts (ET+EX)

Antimicrobial finish with	Zone of inhibition obtained against test microorganism			
	E. coli		*S. aureus*	
	UT+EX	ET+EX	UT+EX	ET+EX
5% w/v leaves extract of *Azadirachta indica* (Neem Tree)	2.53±0.05	2.70±0.1	1.9±0.15	2.36±0.15
5% w/v leaves extract of *Ocimum sanctum* (Holy Basil, Tulsi)	2.4±0.1	2.7±0.1	2.03±0.02	2.23±0.04
5% w/v leaves extract of *Camellia sinensis* (Green Tea)	2.3±0.4	3.03±0.05	2.03±0.4	2.33±0.1

Table V

Percentage reduction of bacteria obtained by modified Hohenstein test for the enzyme-treated and untreated skin samples finished with the 5% w/v natural extracts; UT = Untreated skin, ET = skin with the enzymatic treatment

Test Microorganism	*Ocimum sanctum* extract (Holy Basil)		*Camellia sinensis* extract (Green tea)		*Azadirachta indica* extract (Neem)	
	UT	ET	UT	ET	UT	ET
E. coli	85%±0.05	95%±0.06	88%±0.04	96%±0.03	86%±0.04	97%±0.08
S. aureus	82%±0.02	95%±0.01	81%±0.03	87%±0.05	83%±0.03	92%±0.03

50°C, Treatment time: 60 minutes) goat skin was treated with 5% w/v of leaves extract of *Camellia sinensis* (Green Tea), *Azadirachta indica* (Neem Tree) and *Ocimum sanctum* (Holy Basil, Tulsi). Zone of inhibition obtained against test microorganisms *Escherichia coli* and *Staphylococcus aureus* due to the antimicrobial finish presented in Figure 11. The obtained zone of inhibition was measured, and the details are presented in Table IV.

As seen from Figure 11, untreated and enzyme-treated skin without antimicrobial finish, which was kept as control, showed robust growth of the test microorganisms without an inhibition zone around them. This growth of test microorganisms indicates that untreated and enzyme-treated skin does not exhibit antibacterial activity. However, when untreated goat skin and the enzyme-treated goat skin provided an antimicrobial finish with the natural extracts, a remarkable zone of inhibition was obtained for all three natural extracts against both the test microorganism.

As reported in Table IV, the enzymatically treated and the natural extracts coated goat skin have a high zone of inhibition than untreated and extracts coated goat skin against both the bacteria. Quantitative analysis of antimicrobial finish is evaluated as percentage reduction of bacteria using a modified Hohenstein test, and obtained results are presented in Table V. The results obtained using the modified Hohenstein test agree with the zone of inhibition results. As presented in Table V, a higher value for the percentage reduction of bacteria was obtained in the case of the enzyme-treated skin finished with natural extracts compared to untreated skin finished with natural extracts. This improvement in the antimicrobial finish could be because enzymatic scouring eliminates the non-collagenous substances, resulting in an open skin structure and increasing the antimicrobial agent absorption.

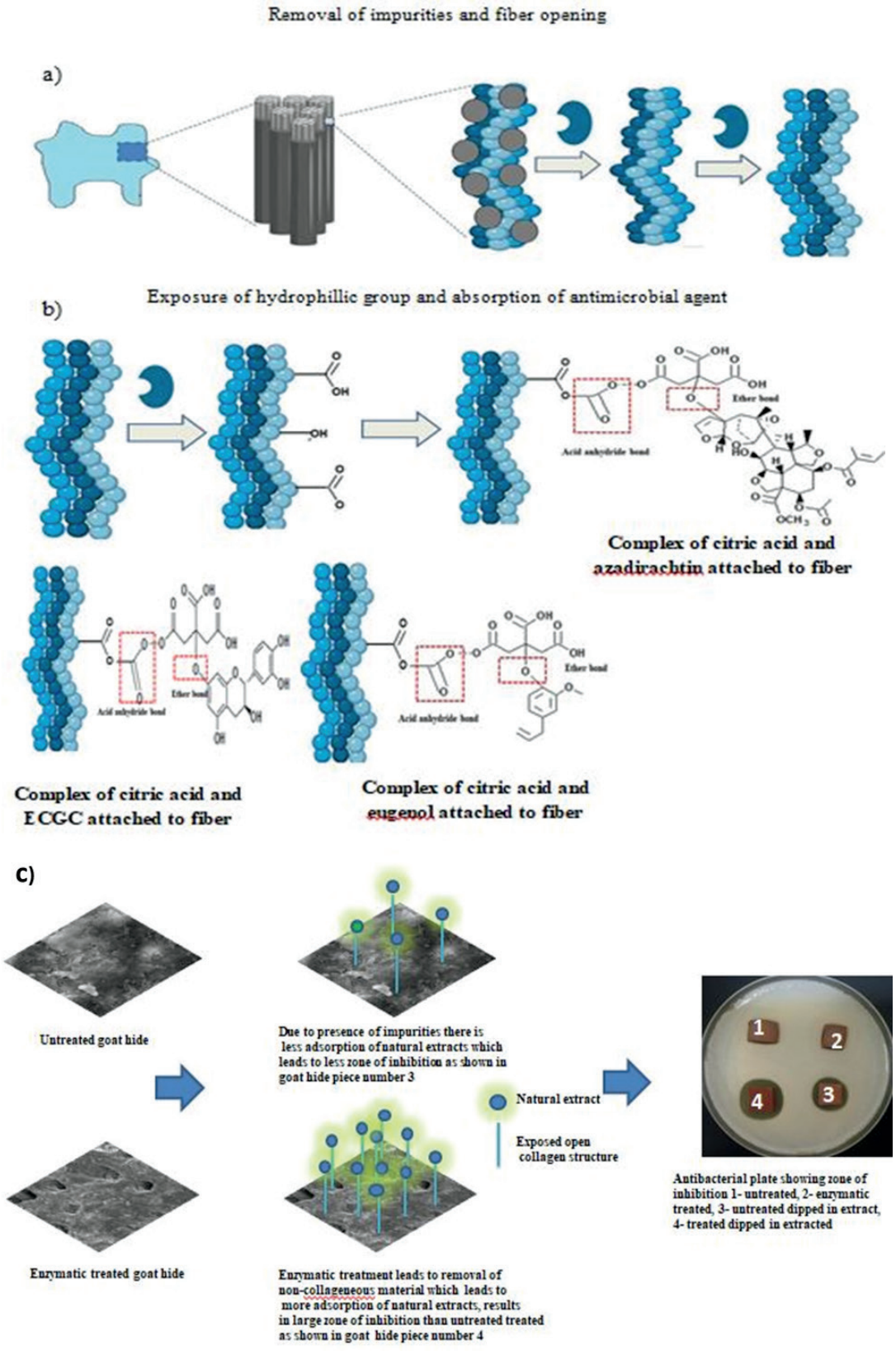

Figure 12. Overall scheme of interaction of treated and untreated goat skin with natural extract

Many scientific studies have alleged that natural plant extract comprises different antimicrobial agents such as flavonoids, terpenoids, polyphenols, tannins, etc.[41] The key ingredients which are responsible for the antimicrobial activity of *Ocimum sanctum*, *Azadirachta indica*, and *Camellia sinensis* leave extracts are eugenol, azadirachtin, and catechin, respectively.[42–47] These antimicrobial agents may significantly alter the lipid layers of bacterial cells or produce morphological changes that inevitably alter the cell membrane's porosity and cause cell degranulation.[47,48]

Skin/leather is a material with variations within the three-dimensional matrix of collagen fibrous weave, which provide a unique nature and governs penetration/uptake of an antimicrobial agent or any finishing agent utilized to impart specific properties. Due to the skin/leather matrix variation, antimicrobial agent penetrates to lesser or greater depth and in varying proportion. Thus, skin/leather provides different affinities and accessibility for antimicrobial agent/finishing agents, and affinity very much depends on the structure and state of skin/leather. The interfibrillar cementing substances pack the collagen fibers, make them hydrophobic and prevent the diffusion of various agents that need to impart new quality to the leather. This study observed a significant decrease in non-collagen components uronic acid and hexosamine with a slight increase in sulfated glucosamine glycan (GAG) after the enzymatic treatment with an acid protease, as reported in Table III. This decrease in non-collagen components compared to control skin after the enzymatic treatment results in the opening of collagen fiber bundles and modification in the three-dimensional matrix of collagen fibrous weave, thus improving wettability and organoleptic properties of the enzyme-treated skin as reported under section 4.7 and 4.8 respectively.

This opening could further accelerate the penetration of the acid protease enzyme through the collagen matrix to act upon anchoring the interfibrillar component. The protease enzymes are known for hydrolyzing the interfibrillar non-collagenous skin components and loosening the collagen weave matrix. Different studies reported similar results with a different class of protease enzyme.[16,49] As revealed from the SEM analysis, the grain surface of skin treated with the acid protease enzyme appeared smoother, uniform, and more homogeneous with observed pores for hair follicles compared to untreated goat skin. The chemical composition of the grain surface of the skin changed after the acid protease treatment without much effect on the bulk properties of the skin. The changes in the chemical composition of the skin after the acid protease treatment studied by ATR-FTIR analysis and XPS indicate an increase in oxygen-containing functional groups at the grain surface as reported in sections 4.2 and 4.3; the XPS study showed an increase in O/C ratio after the enzymatic treatment.

Further, in the XRD analysis, an increase in amorphous properties was observed after the enzymatic treatment of goat skin. These changes in surface chemical composition and decrease in the crystalline fraction of the skin due to the enzymatic treatment provides better affinity and accessibility for the antimicrobial agent present in the natural antimicrobial extracts. The schematic diagram for the interaction of the natural antimicrobial extracts with untreated and enzyme-treated goat skin is presented in Figure 12. As presented in Figure 12, the enzymatic treatment with acid protease enzyme increase the affinity and accessibility of active antimicrobial components of the natural extracts at a time, provides more functional sites for interaction of active components present in these natural extracts, and better attachments due to electrostatic attractions and hence better antimicrobial property.

It can be inferred that the acid protease treatment of goat skin has improved wettability due to the removal of non-collagen components and matrix opening, which eventually led to better absorption of the natural extracts and, thus, enhanced antimicrobial activity. The experiments' results thus allude to the potential of enzymes for environmental-friendly skin processing/leather production. Using enzymes and natural antimicrobial extracts for skin/leather processing would be a privilege for the leather industry workers and the environment, with the ability to replace hazardous chemicals used for skin preservation/tanning operations and provide antimicrobial properties.

5 Conclusions

In this study, goat skin at the pre-tanning stage was treated with the acid protease enzyme; treatment variables such as treatment time, temperature, enzyme concentration, pH, and MLR were studied to optimize the treatment. It was observed that the enzymatic treatment has a profound effect on the goat skin's physical and chemical structure, resulting in improved organoleptic parameters without affecting mechanical strength. The acid protease treatment of goat skin resulted in the removal of non-collagen components from the skin without much damage to the collagen fibrous matrix structure of the skin as conformed with ATR-FTIR spectroscopy, TG/DTA analysis, XRD and SEM characterization, and mechanical strength analysis. The enzymatic treatment also resulted in significant improvement in wettability as observed by measurement of the water contact angle at the grain surface, which reduced to 45.6°±1 after the enzymatic treatment

from 135°± 0.5 for untreated skin. Because of these modifications in the chemical and physical properties of the enzyme tread goat skin, the enzyme-treated skin has better affinity and uptake for the natural antimicrobial extracts obtained from leaves of *Camellia sinensis* (Green Tea), *Azadirachta indica* (Neem Tree) and *Ocimum sanctum* (Holy Basil, Tulsi) and thus better antimicrobial finish which tested against test microorganism *Escherichia coli* and *Staphylococcus aureus*. The acid protease treatment can be utilized as an environmentally friendly alternative to conventional wet chemical treatment to improve antimicrobial finish; the use of enzymatic treatment combined with natural extracts renders an overall environment-friendly antimicrobial leather/leather product.

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Competing Interests

The authors declare that they have no conflict of interests.

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