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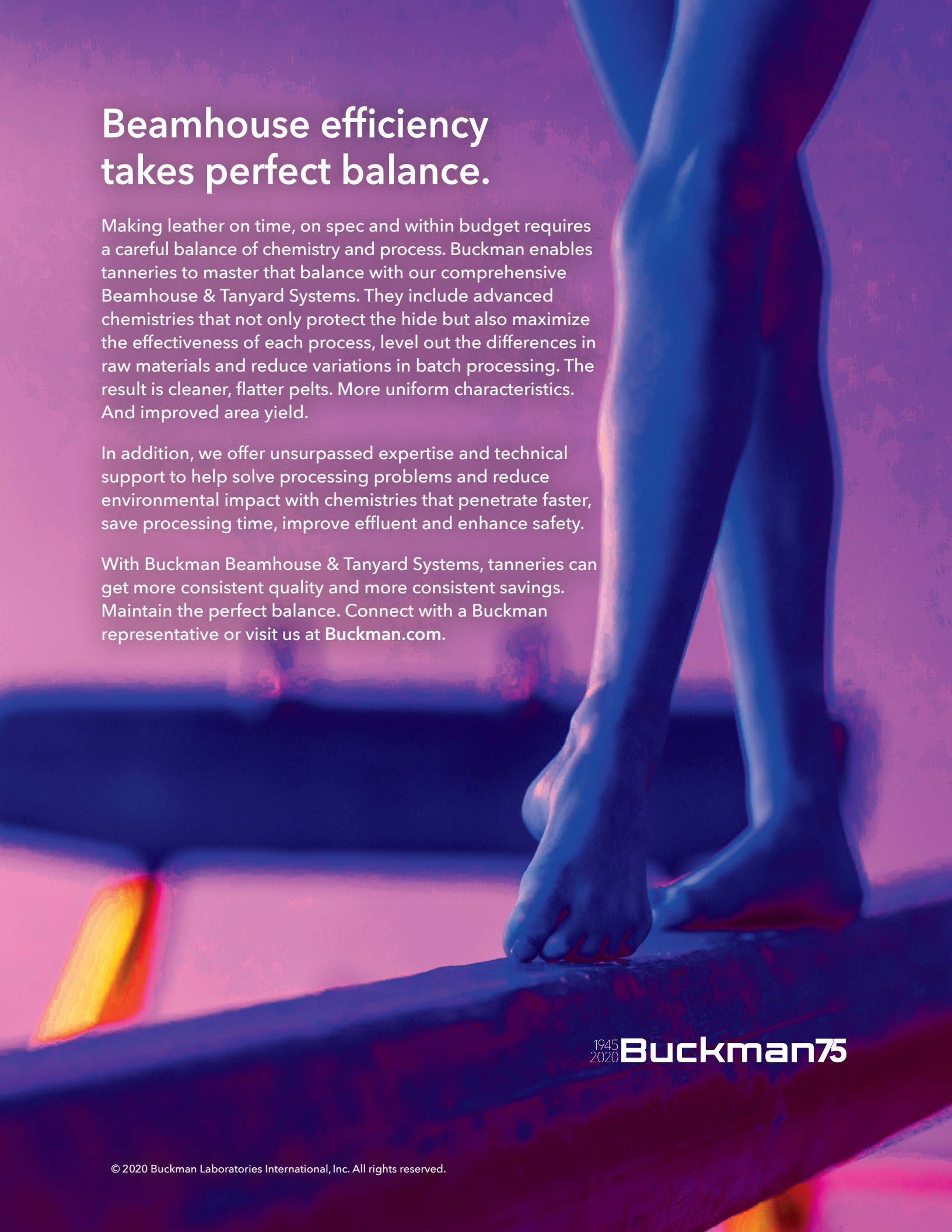

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Transglutaminase Crosslinked Gelatin Films Extracted from Tanned Leather Waste

by

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Abstract

The production of biodegradable polymers has proved to be a promising alternative, since these materials have accelerated degradation, contributing to the reduction of residues and the reduction of environmental pollution. The tannery wastes contain considerable collagen and can be used for gelatin extraction and film production for use in agriculture. Gelatin-based films, however, present some challenges for practical application, such as permeability and solubility in water, parameters that can be improved through the crosslinking process by employing enzymes, promoting the union of polymeric gelatin chains. In this context, the action of the enzyme transglutaminase was investigated to improve the properties of gelatin films recovered from leather and chitosan residues, which were evaluated according to thickness, solubility, permeability, mechanical properties, and soil degradation. The results indicated that the enzyme concentration in the films had a significant effect on the properties of water permeability and solubility and strain to rupture. The evaluation of soil degradation showed that films with higher enzyme addition took longer to be degraded.

Introduction

The polymers from non-renewable sources, despite the intense use in various sectors, such as for packaging production and agriculture, generate high volumes of waste, causing environmental impact due to the time they need to degrade. Biodegradable polymers become an alternative, since they are produced from renewable sources and have faster degradation kinetics, being considered environmentally correct.[1]

Brazil has the potential to use agro-industry waste, since large amounts of waste of animal and vegetal origin accumulate daily, causing problems with logistics and disposal of these materials.[2,3] Among the challenges related to technology to produce biodegradable materials, we highlight the reuse of hazardous waste, as the residues of chromium (III) tanned leather, generated by the tannery industries, which contain considerable protein content from animal hide collagen, being considered by the Brazilian standard ABNT NBR 10.004[4] as Class I - dangerous, due to their toxicity according to the leaching test ABNT NBR 10.005.[5]

There is a growing interest in the process of extracting collagen/gelatin and its derivatives due to the tendency to use this protein in place of synthetic compounds.[6] Gelatin is the product of denaturation and partial hydrolysis of collagen chains, which is an insoluble protein found especially in the skin and cartilage of cattle, pigs and fish, if used as a gelling agent for the development of polymeric films.[7] Gelatin stemming from residues of leather tanned with trivalent chromium has its applicability limited in the food area, due to the possible residual chromium, but promising for applications in agriculture.[8]

Gelatin-based films have good mechanical, optical and sensory properties, although they are sensitive to moisture and have high water vapor permeability due to their hydrophilic character. The ways to reduce the permeability and solubility in water of gelatin films is through the crosslinking process, which promotes the union of two or more polymeric chains.[9, 10, 11] The reticulation of gelatin by the enzyme transglutaminase can catalyze acyl group transfer reactions by forming intra and intermolecular crosslinks in proteins, peptides and various primary amines, mainly through covalent bonds between glutamine and lysine residues. This type of treatment, besides promoting a decrease in the hydrophilic character of gelatin films, also improves the mechanical properties of the material.[12]

In this context, the present work aimed to produce polymeric films using gelatin extracted from residues of chromium (III) tanned leather and to evaluate the effects of the transglutaminase enzyme (TGase) on the properties of thickness, permeability to water vapor, solubility, mechanical properties and degradation of the films in soil.

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Materials and Methods

Protein Extraction

Alkaline hydrolysis was used to extract gelatin from chromium (III) tanned leather shavings.[11] The leather shavings was supplied by a local tannery.

The extraction was performed in an orbital agitator (MA 832, Marconi, Brazil) for 6 h, at 70°C and 180 rpm using a ratio of 50 g of leather shavings, 250 ml of water and 2 g of magnesium oxide.[13] Gelatin in aqueous medium was separated from the solution by vacuum filtration. After filtration, gelatin was concentrated in a dialysis membrane (Polyflux®) with capillary configuration for protein concentration and salt removal.

Film Production

The films were produced with concentrated gelatin, glycerol was added as plasticizer, 20% (w/v) on the gelatin mass, with heating at a temperature of 60°C under agitation for 30 min. The enzyme transglutaminase (ACTIVA® YG, Brazil) was solubilized in water, at a concentration of 1 and 3% (w/v) on the gelatin mass. The gelatin solution temperature was reduced to 37°C, enzyme action time was 15 min. Chitosan was added in a concentration of 1% (v/v). Then, 90 mL of the solution was poured into Petri dishes (19.5 × 2.5 cm) and dried in an environment with controlled conditions (23 ± 2°C and 50% humidity of) for 48 h.

Physical Properties

Thickness

The thickness of the films was determined by means of a digital micrometer with a resolution of 0.001 mm (Mitutoyo, Japan). Ten measurements were performed at points located at the ends and in the center of the films and from this, the arithmetic mean expressed in mm was calculated.

Water Vapor Permeability

Water vapor permeability was determined according to ASTM E96-15[14] using the desiccant method. The films were sealed to the nozzle of a test bottle with a diameter of 2.5 cm containing 10 g of silica (4-8 mm) and these were arranged in an environment with humidity maintained at 75%. The weighing was performed every 1.5 h for a total of 11.5 h. The ratio between the variation of the assembly weight and the area of the bottle nozzle was compared with the elapsed time. The slope of the drawn line is the rate of transmission of water vapor. All weight measurements were performed using analytical balance (AUY 220, Shimadzu, Japan). Relative humidity (75%) was controlled using a saturated sodium chloride solution.

Solubility

The percentage of water-soluble material was determined according to the method described by Cuq et al., 1997[15] with some adaptations. Square samples of 2 × 2 cm were dried in an oven (Model A35ED, DeLeo, Brazil), at 70°C for 24 h. Subsequently, they were weighed on an analytical balance (Model AUY220, Shimadzu, Japan) to determine the initial mass. The dried samples were immersed in 50 mL of distilled water and kept under agitation for 24 h at 80 rpm and 25°C in a thermostatic bath (Model 501/1D, Nova Era, Brazil). After this period, the samples were removed from the water and dried for 24 h at 70°C and weighed to obtain the final mass.

Mechanical Properties

The determination of mechanical properties, maximum tensile strength and elongation at break, consists of an average of two repetitions. The tensile tests were performed on a universal machine (model DL 2000, Emic, Brazil). The films were cut into a standard rectangular shape (20 mm × 100 mm) according to the ASTM D882-12.[16]

Degradation of Films

The evaluation of microbiological susceptibility of the films was performed according to ASTM G160-03.[17] The samples were cut in the dimensions of 2 × 2 cm, in triplicate. The soil (40 g) used in this experiment was placed in transparent 200 ml polypropylene (PP) cups, the samples were incubated for up to 12 h. The samples of the films were taken at the times of 1, 3, 8 and 12 h and analyzed by Thermogravimetry and FTIR. Each sample was washed with distilled water.

Thermogravimetry

The films thermogravimetry was performed in a simultaneous thermal analysis instrument (Model Jupiter 449, Netzsch, Germany). Aliquots of 10 mg of the samples, were submitted to a heating rate of 10°C/min in nitrogen atmosphere (50 ml/min) from 20° to 700°C.

Fourrier Transform Infrared Spectroscopy

FTIR experiments were performed on an infrared spectrometer with Fourier Transform (Model Spectrum 400, Perkin Elmer). The equipment was operated in attenuated total reflection mode (ATR) using diamond crystal. Thirty-two scans were performed with a resolution of 2 cm^{-1} in the wavelength range of 450 to 4000 cm^{-1}.

Statistical Analysis

The statistical significance of the factors tested was evaluated by means of Variance Analysis (ANOVA). Statistical analysis was performed with the aid of Statistica Software 12 (StatSoft Inc.). A 95% confidence level was used in all statistical analyses.

Results and Discussion

Physical Properties

The results obtained for each physical property can be visualized in Figure 1. The values were also statistically analyzed to verify if the enzyme TGase had significant effect on the film's properties.

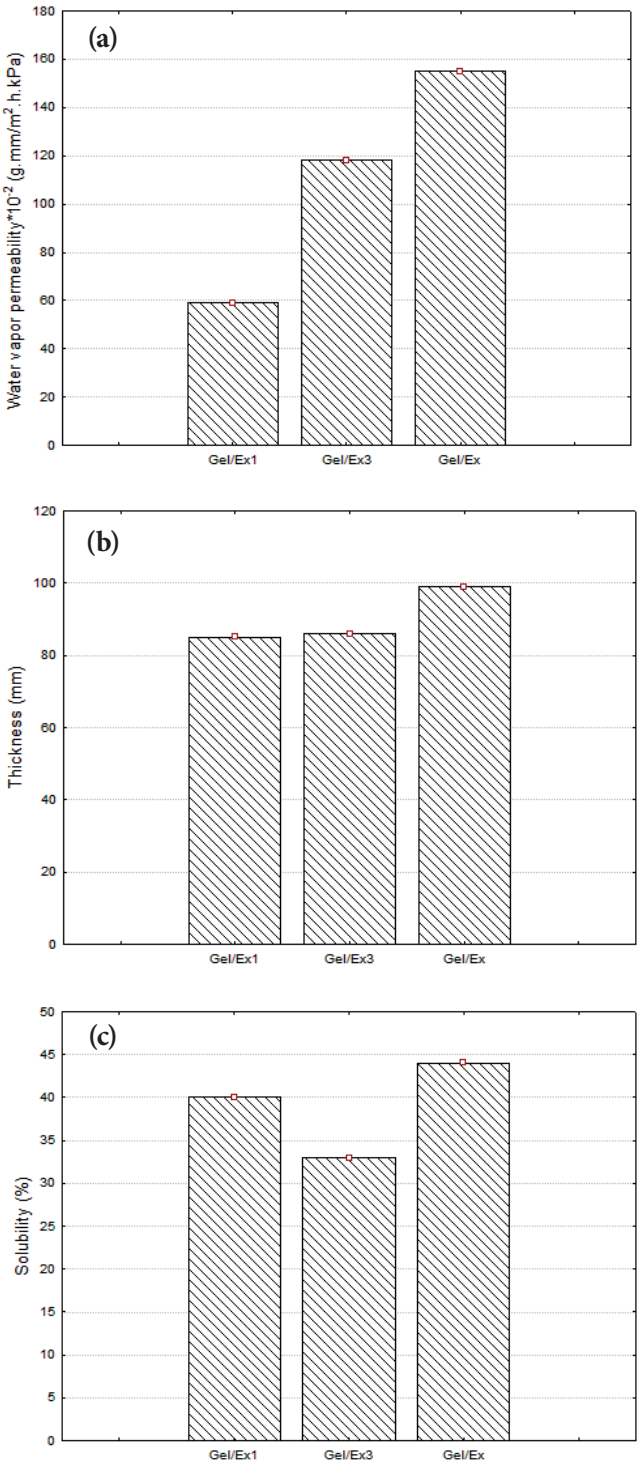

Figure 1. Physical properties of films **(a)** thickness, **(b)** water vapor permeability and **(c)** solubility. Gel/Ex1: Gelatin films with 1% TGase; Gel/Ex3: 3% TGase; Gel/Ex: no enzyme addition.

The statistical analysis indicated that the addition of enzyme had no significant effect on the thickness property (p = 0.72). The film without enzymatic treatment Gel/ Ex has a thickness of 0.099 mm and the films with enzymatic treatment, Gel/Ex1 and Gel/Ex3 have a thickness of 0.085 and 0.086 mm, respectively.

The different concentrations of enzyme showed a significant effect on the property of water vapor permeability (p = 5.05E-5). The permeability of the film without enzymatic treatment (Gel/Ex) was 1.55 g.mm.m^{-2}.h^{-1}.kPa^{-1}, and the films with enzymatic treatment Gel/Ex1 and Gel/Ex3 presented 0.59 and 1.18 g.mm.m^{-2}.h^{-1}.kPa^{-1}, respectively, that is, the enzyme at the concentration of 1 and 3% (w/w) promoted a reduction in permeability compared to the film without enzymatic treatment. This difference can be explained by the crosslinking process, which promotes the union of polymer chains through covalent bonds between the atoms. Thus, TGase reduces the space between the pores of the polymer matrix, making it difficult for the process of diffusion of water through the film.[12, 18] Similar results were observed by Wangtueai, Noomhorn and Regenstein, 2010,[19] who applied lizard-fish gelatin and obtained a permeability value of 26.3 g.mm.m^{-2}.d^{-1}.kPa^{-1} without adding enzyme to the film and, with 0.5% TGase, this value decreased to 21 g.mm.m^{-2}.d^{-1}.kPa^{-1}. The same was observed by Nishihora, Niehues and Quadri, 2015,[9] who worked with pig skin gelatin and obtained permeability values of 0.29 g.mm.m^{-2}.h^{-1}.kPa^{-1} for films without enzymatic treatment and, when adding 12.71 mg.ml^{-1} of TGase to the film, this value decreased to 0.1643 g.mm.m^{-2}.h^{-1}.kPa^{-1}. However, in a study carried out by Kołodziejska and Piotrowska, 2007[20], who used fish skin gelatin, the enzymatic crosslinking of the films did not allow improvement in the properties of barrier to water vapor, since the permeability of films not modified with TGase was 2.42 g.mm.m^{-2}.h^{-1}.kPa^{-1} and for those treated with 0.2 mg.ml^{-1} of TGase, this value was 2.40 g.mm.m^{-2}.h^{-1}.kPa^{-1}. It is highly likely that occurred because of different protein components of the films and the various enzymatic reaction conditions used in the preparation of the films.

On the other hand, when comparing the permeability values obtained in the films with the enzymatic treatment, Gel/Ex1 and Gel/Ex3, it's possible to observe that the increase in the concentration of TGase causes an increase in the permeability of the films, since the film with 1% of enzyme concentration obtained 0.59 g .mm.m^{-2}.h^{-1}.kPa^{-1} permeability and, by increasing this concentration by 3%, the permeability also increased by 1.18 g.mm.m^{-2}.h^{-1}.kPa^{-1}. The same was observed by Nishihora, 2015,[21] who worked with 60 mg.mL^{-1} of swine skin gelatin and 50 mg.mL^{-1} of glycerin as a plasticizing agent and, when adding enzyme in concentrations of 4 and 12 mg.mL^{-1}, obtained films with permeability of 0.7182 and 1.0328 g.mm.m^{-2}.h^{-1}.kPa^{-1}, respectively. This increase can be explained by the presence of plasticizer that, together with the increase in enzyme concentration, generates an increase in the free volume of the structure and, thus, facilitates the diffusion of moisture through the film. In addition, the higher concentration of TGase can cause an increase in the mobility of the chains, which results in a higher coefficient of water diffusion and, thus, greater water vapor permeability.[21, 22]

Statistical analysis indicated that the addition of the enzyme had a significant effect on solubility property (p = 0.023). The films obtained from gelatin without Gel/Ex enzymatic treatment showed solubility

of 44.37% and films with Gel/Ex1 and Gel/Ex3 treatment showed solubility of 40.38 and 33.12%, respectively. A decrease in solubility property occurs with increased enzyme concentration in films. This fact justifies the reduction of spaces in the polymer matrix, which occur due to cross-linking in the protein. Similar results were found by Yayli, Turhan and Saricaoglu, 2017,[23] when evaluating solubility in edible films derived from chicken gelatin treated with different concentrations of transglutaminase. The authors reported that there was a decrease in solubility as an increase in the concentration of TGase. Films treated with 1 and 3% enzyme obtained solubility values of 36.37% and 32.14%, respectively. Liu et al., 2017[24] studied bovine gelatin films treated with 0.6% TGase (m/m) and glycerol, dried at different temperatures. It was observed that as the drying temperature increased (from 15° to 35°C) there was a decrease in solubility in the films. The values for this property ranged from 36.7 to 34.4%.

Mechanical Properties

Gelatin films were characterized for mechanical properties, tensile strength at break and elongation. Figure 2 shows the results.

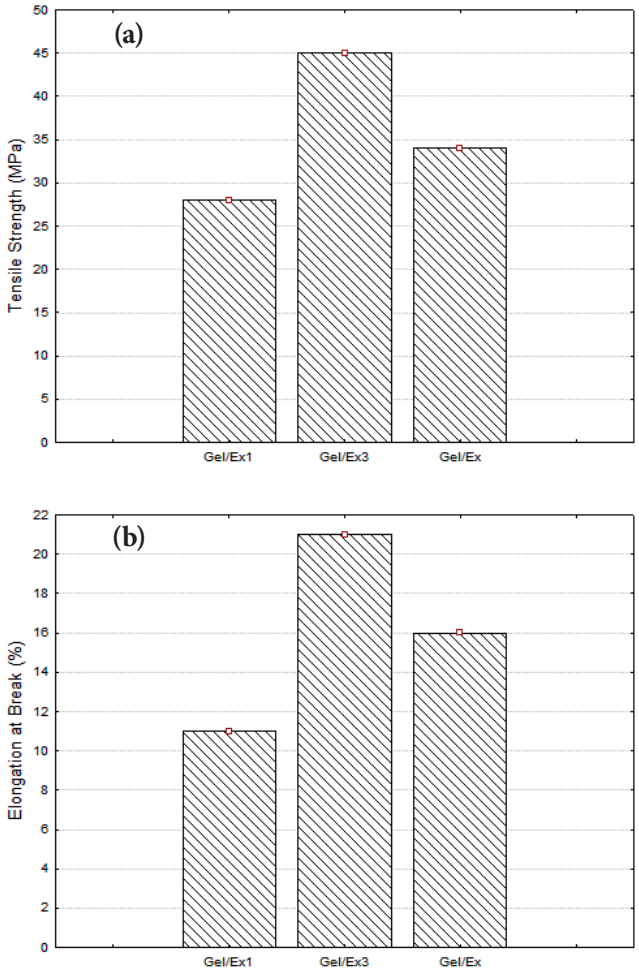

Figure 2. Mechanical properties of **(a)** tensile strength and **(b)** elongation at break of films obtained from recovered gelatin. Gel/Ex1: Gelatin films with 1% TGase; Gel/Ex3: 3% TGase; Gel/Ex: no enzyme addition.

The statistical analysis showed a significant difference for the tensile strength property of the films ($p = 0.028$), while for the elongation at break property, the statistical analysis did not present a significant value ($p = 0.109$).

The increase in the tensile strength for the film with the highest concentration of enzyme occurs due to the greater crosslinking between the gelatin molecules, acting as a reinforcement between the bonds of the matrix structure, configuring greater rigidity and less flexibility of the polymer.[18, 25] The mixture between chitosan and gelatin can also cause an increase in the mechanical properties due to interactions between electrostatic and hydrogen bonds, forming a more stable network between the polymers and, consequently, increasing the rigidity of this one.[26]

Masamba et al., 2016[27] analyzed zein films treated with different concentrations of transglutaminase and oleic acid. The concentration of 1% of TGase showed the highest value for tensile strength (26.9 MPa). Still, the authors found that low or high concentrations of enzyme in the formulation of the films can negatively affect the tension and elongation properties.

Jridi et al., 2014[28] and Hosseini et al., 2012[29] observed that films composed with fish gelatin and chitosan, plasticized with glycerol, had their tensile strength values increased as the chitosan content in the composition of the films increased. However, the values for elongation decreased as the presence of chitosan increased. The mixing of the two polymers caused the crosslinking of the polymeric matrix, where chitosan acted as a plasticizer, weakening hydrogen bonds and making the films more flexible.

A control film was produced with commercial gelatin, without the addition of an enzyme, which resulted in a tensile strength and elongation at break value of 65.5 MPa and 20.41%, respectively. The tensile strength is about 92% higher for commercial gelatin films when compared to gelatine recovered from residues, both without the addition of enzyme. It is observed that the addition of the enzyme has a positive effect, since when 3% of the enzyme is added to the formulation with extracted gelatin (Gel/Ex3), the rupture stress increases around 32% (34 MPa to 45 MPa), however the commercial gelatin film without addition of enzyme still has a tensile strength about 45% higher. The tensile strength in commercial gelatin films tends to be higher than in extracted gelatin films, since in the latter there is a greater presence of salts in their composition, which can cause the matrix to swell due to the hygroscopicity associated with minerals, thus causing an increase of the mobility of the polymer chains and weakening the bonding forces between them which, consequently, causes an increase in the deformation capacity and a decrease in the breaking force.[30]

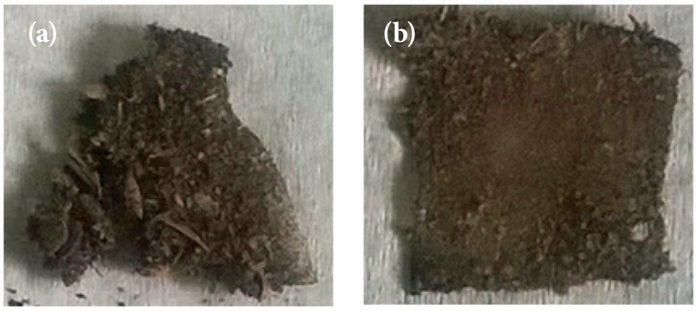

Figure 3. Films produced with gelatin extracted from chromium (III) tanned leather, with **(a)** 1% of TGase and **(b)** 3% of TGase, after 12 h of exposure into the soil

Films Degradation Assays

The evaluation of the microbiological susceptibility of the films was carried out according to the ASTM G160-03[17] standard.

In the present work it was not possible to determine the mass loss of the produced films, as the soil adhered to the material in the first hour of exposure and, consequently, the washing step for later weighing could not be performed. From Figure 3, the films are observed after 12 h of exposure into the soil.

The film without the addition of the enzyme transglutaminase (Gel/Ex) was already in an advanced stage of degradation after 12 h of exposure, and it was not possible to take photographs, as the degradation occurred before the first removal of samples from soil. The film with 1% of enzyme (Gel/Ex1) presented signs of degradation, while the film with 3% of transglutaminase enzyme (Gel / Ex3) was more intact. Thus, it was possible to observe that the increase in the enzymatic concentration gives greater integrity to the polymeric structure of the material, reducing the degradation when exposed to soil. Both films were completely degraded after 24 h of exposure to the soil.

In a study carried out by Lucena et al., 2017,[31] it was revealed that films produced with xylan and gelatin have high degradability (less than 15 days) and can be considered as a new raw material that is primarily non-polluting in industry.

Thermogravimetric Analysis of the Films Removed from Soil

For the Gel/Ex and Gel/Ex1 films, it was not possible to perform the thermogravimetric analysis, due to the soil adhered to the samples. Figure 4 reveals the thermogravimetry of Gel/Ex3 samples collected at different exposure times in soil.

The first stage of degradation is related to the beginning of the evaporation of more volatile compounds, such as free water present on the surface of the film. The second stage occurs due the decomposition of more complex molecules, such as polysaccharides and proteins, represented in these films by chitosan and gelatin, respectively. The last stage refers to the degradation of bonds still present in the polymeric matrix.[32]

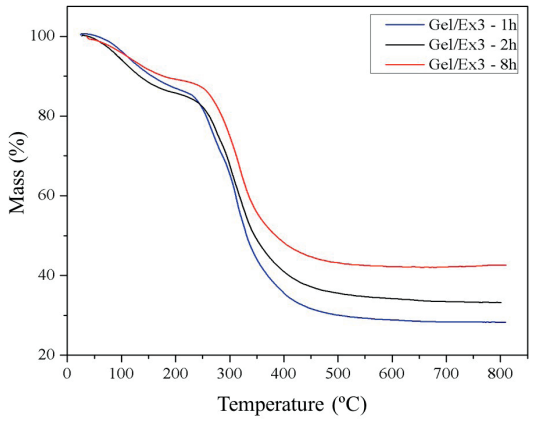

Figure 4. Thermogravimetry for films of extracted gelatin with 3% of enzyme (Gel/Ex3), after 1, 3 and 8 h of exposure in soil.

The residual mass content can be justified by two factors (1) because it is a film produced from gelatin extracted from leather residue, there may be the presence of salts from the tanning of the hides still attached to the protein molecule, and (2) the longer the exposure time to the soil, the ash content also increases, justified by the adhesion of organic and inorganic matter to the film and the formation of new products linked to the polymeric chain.[32]

FTIR from the Films Exposed to the Soil

Figures 5a and 5b show the FTIR spectra for films produced with gelatin extracted from chromium (III) tanned leather shavings, arranged in soil for the periods of 1 and 3 h.

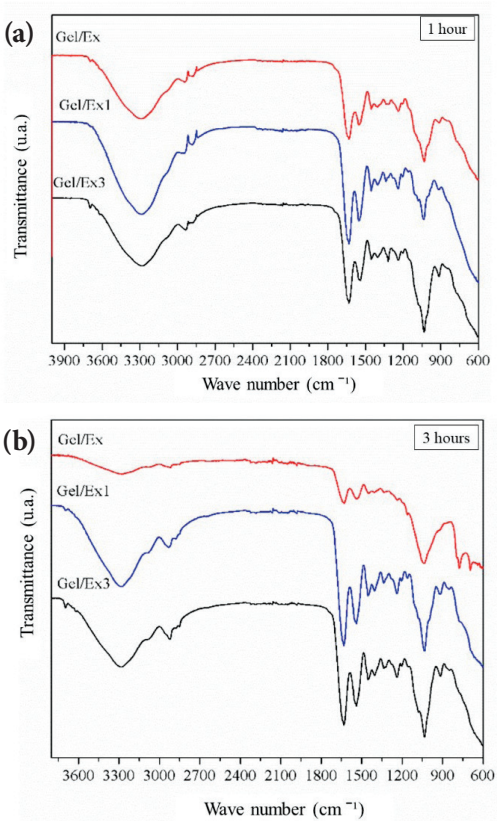

Figure 5. FTIR curves for extracted gelatin films (Gel/Ex, Gel/Ex1 and Gel/Ex3) after **(a)** 1 h exposure to soil and **(b)** 3 h exposure to soil. Gel/Ex1: Gelatin films with 1% TGase; Gel/Ex3: 3% TGase; Gel/Ex: no enzyme addition.

According to the FTIR graphs, it is observed that the peak corresponding to amide I (~ 1650 cm^{-1}) is reduced in intensity after 3 h of exposure to the soil (Figure 6b) for the film without enzymatic treatment (Gel/Ex). This peak represents the stretching of C = O (carbonyl) bonds that can be defined as an observation peak to check the degradation of a polymer due to the break in the bond between carbon and oxygen.

The decrease in vibrational bands for -OH (~ 3500 cm^{-1}) and -NH (~ 3600 and 1650 cm^{-1}) molecules indicates that there is a weakening of hydrogen bonds between the polymers that form the film due to the absence of an agent, such as the enzyme transglutaminase, which is able to stabilize chemical bonds between materials. The enzyme provides a stronger intermolecular interaction between the reactive groups and its absence in the films can cause the rupture of the polymeric network due to the action of external agents such as humidity and the action of microorganisms.[24, 29, 34, 35]

For the films with enzymatic treatment (Gel/ Ex1 and Gel/Ex3) it is noted that the peaks corresponding to the -OH stretch of water molecules and -NH stretch in wave number ranges between 3000-3600 cm^{-1} increase after three hours of exposure to soil. These observations indicate that the addition of transglutaminase enzyme interfered with the formation of hydrogen bonds between the -OH and -NH groups, demonstrating that there were changes at the molecular level when the enzyme and the polymer matrix interacted.[36] The absorption band present at 3300 cm^{-1} is due to the presence of glycerol -OH bonds that are expressed in this spectrum range, and to the absorption of water by the films, which have hygroscopic characteristics. In addition, the presence of glycerol can be confirmed by the presence of the peak close to 1080 cm^{-1}, typical of the interaction between the -OH group and gelatin through hydrogen bonds.[26]

In the range from 1235 to 1541 cm^{-1}, typical bands of gelatin samples are present, representing amine and amide groups (secondary and tertiary). The absorption band at 1541 cm^{-1}, frequently used in the identification of proteins, stands out.[37]

The FTIR analysis and the results obtained by visual analysis of the samples after three hours of exposure to the soil show that the enzyme was effective from the point of view of increasing the resistance to the degradation of the films, in addition to having contributed effectively to the increase of important mechanical properties.

Conclusion

Films produced from protein extracted from chromium III tanned leather residue had their properties improved with the addition of the enzyme TGase.

The tests indicated that the TGase acted in the films reducing the thickness, the amount of water vapor permeability and the solubility of the films. The mechanical properties such as elongation and rupture stress were increased for films with higher concentration of TGase enzyme. The degradation test in simulated soil proved that the films are biodegradable and that the addition of the enzyme transglutaminase promoted the crosslinking of the polymer matrix, maintaining the integrity and degradation of the films for a longer time.

Futhermore, biodegradable gelatin films are a promising alternative to materials produced from non-renewable sources. The films produced can be used as soil cover, providing essential nutrients, due to the presence of nitrogen in its polymer structure, in addition to contributing to the environment, with the reduction of the environmental impact caused by the slow decomposition of polymers.

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Ionic Liquid Functionalised Nanoparticles Based Tanning System as a Less Chrome Tanning Approach

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Abstract

Nanoparticles due to their size and high reactivity towards collagen provide potential applications in the tanning process. The current study aims to investigate the potential of ionic liquid functionalized cerium oxide nanoparticles as a combination tanning agent with minimum utilization of the chromium. The experimental results indicate that the presence of nanoparticles increases the thermal stability from 62°C to 92°C. The physical strength and organoleptic characteristics of the nanoparticles-BCS tanned leather are on par with conventional chrome tanned leather. Antibacterial studies reveal that the leather tanned with nanoparticles shows improved antibacterial activity. Thus, nanoparticle based tanning system invokes a considerable array of interest as an alternative tanning process with minimal and efficient usage of chromium.

Introduction

Tanning is the most crucial unit operation in leather processing, which provides persistent stability to the skin thereby protecting it against microbial invasion.[1] Chromium and vegetable tannins are most commonly used for the tanning process.[2-5] Between them, 90% of globally produced leathers receive chromium in some form.[1] Despite all the technical advantages chrome tanning has its own limitations as well and hence there is thrust towards less chrome approach.[5] One such alternative process involves the usage of nanoparticles for tanning. Nanoparticles could easily penetrate inside the interfibrillar spaces and have significant interaction with the carboxyl and amino side chains present in collagen,[6-8] which results in the improved mechanical properties and hydrothermal stability of collagen.

Cerium oxide exists commonly in oxidation state Ce^{3+} and Ce^{4+} and is known to interact with biomolecules.[9] Cerium oxide nanoparticles find greater application in metal oxide fuel cells and in biomedical application such as a carrier for targeted drug delivery[10-12] and because of the catalytic regenerative antioxidant property they are mainly found to be effective against bacterial species.[13,14] Capping agent used in the conventional nanoparticles synthesis to prevent agglomeration of nanoparticles and stabilization process are mostly organic. To overcome these limitations, ionic liquids, the designer green solvents have been used for the synthesis of nanoparticles. Choline based ionic liquids was finds application in the enhancing the thermal stability of the collagen based biomaterials.[15,16] Thus ionic liquid can act as a both capping agent and provide functionalities to the nanoparticles. In recent times, imidazolium based ionic liquid has been used in unhairing and fiber opening processes for cleaner leather processing.[17] Thus, it is essential to prepare a nanoparticle with mild eco-friendly reagents.

Ionic liquid functionalized cerium oxide nanoparticles have been reported recently by our group to enhance the thermal stability of collagen at the fiber level.[18] In the present study, tanning of leathers using these nanoparticles has been optimised and the leathers have been evaluated for physical strength characteristics and antimicrobial activity against bacterial species.

Experimental Section

Materials

Goat skins were used as the raw material for all the leather trials. All the chemicals used for leather processing were of commercial grade.

Methods

Synthesis of Cerium Oxide Nanoparticles

Cerium oxide nanoparticles was synthesized from the earlier method.[18] In which cerium nitrate (III) precursor and sodium hydroxide was added to 2 mL of the choline serinate IL followed by stirring for 45 min. The mixture was kept in a sonication bath for 12 hours. The final product was centrifuged and washed repeatedly with solvent and water followed by overnight drying in hot air oven. The hydrodynamic diameter, zeta potential and, polydispersity index was found to be 192.3 ± 2.14 (d.nm), -13.76 ± 1.5 (mV) and 0.387 respectively.

Pretanning Leather Process

Goat skins were processed by the conventional pretanning unit operations. Wet salted skin was soaked followed by unhairing, fiber opening, fleshing, deliming and pickling process. Pickled pelts were used for the trials.

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Optimization of Nanoparticles Input

The conventionally processed pickled pelts were treated with five different percentages of nanoparticles viz 5%, 4%, 3%, 2% and 1% in order to optimise the nanoparticles offer. In tanning, water was used as the medium. Followed by this, 2% Basic Chromium Sulphate (BCS) was used to further enhance the thermal stability of the leather.

Analysis of Tanning Liquor

Tanning liquors from the conventional tanning and Nanoparticles-BCS combination processes were subjected to chromium exhaustion and chrome content analysis as per standard procedure.[19] Measurements were carried out in triplicate and average values are reported.

Analysis of Thermal Stability

A SATRA STD 114 shrinkage tester was used for the analysis of thermal stability of tanned leather. A post tanned leather piece of about 3 cm × 1 cm was cut and tied to one end by means of a steel hanging and allowed to immerse in glycerol water mixture (3:1). The temperature of the water bath was increased from 40°C to 120°C. The temperature at which the leather visibly shrinks in the water bath is noted and it is identified as the shrinkage temperature. Measurements were carried out in triplicate and average values are reported.

Physical Test Characteristics

Nanoparticles tanned leather was subjected to physical testing, sampling for analysing tensile strength and tear strength according to standard procedures.[20] Measurements were carried out in triplicate and average values are reported.

Evaluation of Anti-Bacterial Property

Bacillus subtilis and Staphylococcus aureus bacterial strains obtained from MTCC were used to evaluate the antibacterial activity. The bacterial stock cultures were incubated for 24 hours at 37°C on nutrient agar and stored at 4°C. The 100 µl of the bacterial culture was inoculated on the Miller-Hinton agar plates; conventional chrome tanned leather was used as a control. Nanoparticles-BCS combination and conventional tanned leather were cut into small pieces and placed on the agar plate and incubated at 37°C for overnight, followed by measurement of the zone of inhibition.

Morphological Analysis

Tanned leather obtained from the conventional and nanoparticles-BCS combination tanning processes were cut from a sampling position with uniform thickness and gradually dehydrated as per the standard acetone dehydration procedure. All specimens were then coated with gold using a Palaron range CA7620- sputtering coater. A FEI Quanta 200 scanning electron microscope and energy dispersive X-Ray was used in order to study the morphological characteristics and elemental composition of the leather.

Table I

Optimization of nanoparticles offer

Nanoparticles Input (%)	Shrinkage Temperature (°C)
1	68 ± 1
2	69 ± 1
3	71 ± 1
4	73 ± 1
5	73 ± 1

Estimation of Cerium Oxide

The amount of cerium in the effluent was determined by spectroscopic method using uv-visible spectroscopy. Different concentrations of cerium were used as standards. A known concentration of cerium was taken in 10 ml of sulphuric acid, 0.5 mg of silver nitrate and 24 mg of potassium persulfate. Solution was boiled for 5 to 10 mins and the solution cooled for 5 mins and absorbance measured at 320 nm.[21]

Results and Discussions

Effective Nanoparticles Input

In order to find the effective concentration of nanoparticles, five different concentrations of nanoparticles were taken for tanning process. Thermal stability has been an essential factor in the tanning process. Table I shows 1%, 2% and 3% nanoparticles tanned leather were hydrothermally stable up to 68°C, 69°C and 71°C, respectively. Whereas in the case of 4% and 5% nanoparticles offer thermal stability increased to 73°C. As both inputs showed similar stability, we fixed the nanoparticles concentration as 4% offer.

Nanoparticles-BCS Combination and Conventional Tanning System

Poor exhaustion of chrome (about 60 to 70%) is one of the limitations in the conventional chrome tanning system. Ions present in ionic liquids are commonly known as kosmotropes and chaotropes. The kosmotropes are structure maker and chaotropes are structure breaker. The choice of ions plays an important role in determining the functional property of the ionic liquids. The kosmotropic anion and chaotropic cation are known to stabilize the protein, whereas the kosmotropic cation and chaotropic anion, destabilizes the protein. In this study, we use chaotropic cation (choline) and

Table II

Combinational and conventional tanning process

Process	Chemicals	(% Offer)	Time	Remarks
Washing	Water	100	10 mins	
Deliming	Water	100		Completion was checked by Phenolphthalein
	Ammonium Chloride	3	60 mins	
Washing	Water	100	10 mins	
Bating	Water	100		Air bubble to check the completion
	Microbate-R	0.5	60 mins	
Washing	Water	100	10 mins	
Pickling	Water	100		Check the pH 2.8-3.0 and drain ⅓rd of pickle liquor
	Sodium Chloride	10		
	Water	10		
	Sulphuric Acid	1	4×5 60 mins	
Tanning				
Control	Water	50		Check cross section for penetration
	BCS	8	60 mins	
Experiment	Water	50		Check cross section for penetration
	CE-NP	4		
	BCS	2	60 mins	
Basification	Sodium Formate	1		Check the pH 3.8-4.2 and ageing for 24 hours
	Water	10		
	Sodium bicarbonate	1	4×5 60 mins	

kosmotrpic anion (serinate) for functionalizing nanoparticles. Since serine, a polar amino acid is the most reactive group in the choline based ionic liquids;[15] they could form electrostatic linkage as an amino group in serinate coated on the cerium oxide nanoparticles interacts with the carboxyl side chains of collagen thereby creating additional sites for the interaction of chromium as both form competitive sites with collagen. From Figure 1 it is evident that the nanoparticles-BCS combination tanning discharge liquor is almost colorless, whereas the conventional tanning discharge liquors look dark blue in color and concentration of cerium oxide nanoparticles was found to be 10 mg/ml. As mentioned in Table III, the chrome content of 8% BCS discharged waste liquor was relatively higher and darker compared to the nanoparticles-BCS combination tanning process.

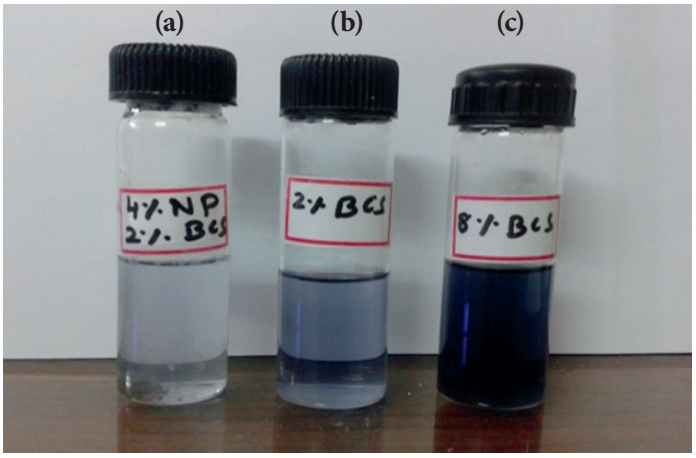

Figure 1. Spent tanning liquor **(a)** Combination tanning **(b)** 2% BCS tanning **(c)** Conventional tanning

Table III

Characterization of conventional and Nanoparticles-BCS combination tanning process

Characteristics	Conventional tanning	2% BCS Tanning	Combination Tanning
BCS input (%)	8	2	2
Nanoparticles input (%)	–	–	4
Chromium uptake	68 ± 2	92 ± 1	94 ± 1.5
Shrinkage temperature (T_s°C)	118 ± 2	77 ± 2	92 ± 1
Chrome content of waste liquor (mg/L)	2200 ± 10	66± 3	57± 2

Morphological Studies

The effect of different concentrations of nanoparticles input on the tanned leather was studied by the cross-section micrographs at a magnification of 100X and the images are shown in Figure 2. It is evident from the SEM images that the increasing concentration of nanoparticles from 1% to 4% has an influence on the penetration of chromium into the leather fibers. The fiber orientation and compactness of the 4% nanoparticles and 2% BCS tanned leather is similar to that of conventionally tanned leather. These results indicate that the nanoparticles do not affect the morphological characteristics of nanoparticles-BCS combination process tanned leather. The elemental composition of the tanned leather was aligned with the combinational tanning process.

Physical Strength Characteristics

The effect of nanoparticles on the physical strength characteristics of the tanned leather was studied. Crust leather was made from nanoparticles-BCS combination and conventional tanning processes, followed by conventional retanning, fat liquoring, dyeing process and other mechanical operations were further subjected to physical strength and organoleptic characteristics as shown in Table IV and V. There is a minimal decrease in strength and organoleptic characteristics of nanoparticles-BCS tanned leather compared to the conventional tanning process, which is due to the lesser percentage of the chromium in the nanoparticles tanning process.

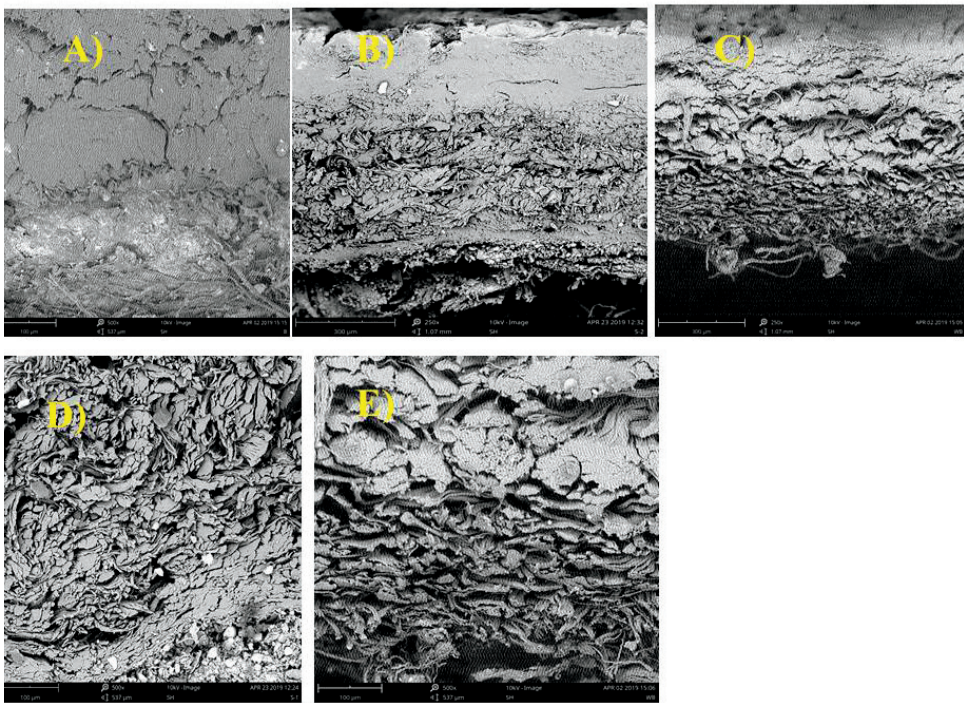

Figure 2. Scanning electron micrographs showing cross-section of various nanoparticles input of tanned leather **(A)** 1% NP- 2% BCS, **(B)** 2% NP- 2% BCS, **(C)** 3% NP - 2% BCS, **(D)** 4% NP - 2% BCS, **(E)** Conventional tanning process

Table IV

Physical strength characteristics of conventional and combination tanning

Characteristics	Conventional Tanning	Combination Tanning
Tensile strength (N/mm^2)	15.75 ± 3.76	12.14 ± 1.52
Elongation at break (%)	45.75 ± 3.65	40.13 ±2.21
Tear Strength (N)	32.92 ± 2.2	28.48 ± 1.38
Load at grain crack (kg)	33 ± 2	29 ± 1

Table V

Organoleptic properties characteristics of conventional and combination tanning

Properties	Conventional Tanning	Combination Tanning
Softness	8/10	7/10
Tightness	8/10	6.5/10
Roundness	7/10	6/10
Smoothness	8.5/10	7/10

Anti-Bacterial Studies

The inhibitory activity of cerium oxide nanoparticles-BCS combination tanned leather was studied in Gram-positive bacterial culture since their cell wall has a thick outer membrane compared to the gram-negative bacteria. The antioxidant property of cerium oxide nanoparticles induces oxidative stress and forms reactive oxygen species on the cell membrane of microorganism thereby rupturing the cell membrane and inhibiting growth of the microorganism.[13] As shown in Figure 3 zone of inhibition of *Bacillus subtilis* and *Staphylococcus aureus* was found to be 25±1 and 23±2 mm, respectively.

Conclusions

In summary, cerium oxide nanoparticles were synthesized by sonication method and controlled reaction condition resulted in the formation of nanoparticles 20 nm diameter. The application of the nanoparticles for tanning has been studied along with minimal concentration of chromium. It has been evident ionic liquid coated nanoparticles enhance the wet heat resistance temperature and absorption of chromium in the nanoparticles-BCS combination tanning system. Nanoparticles also improve the anti-bacterial

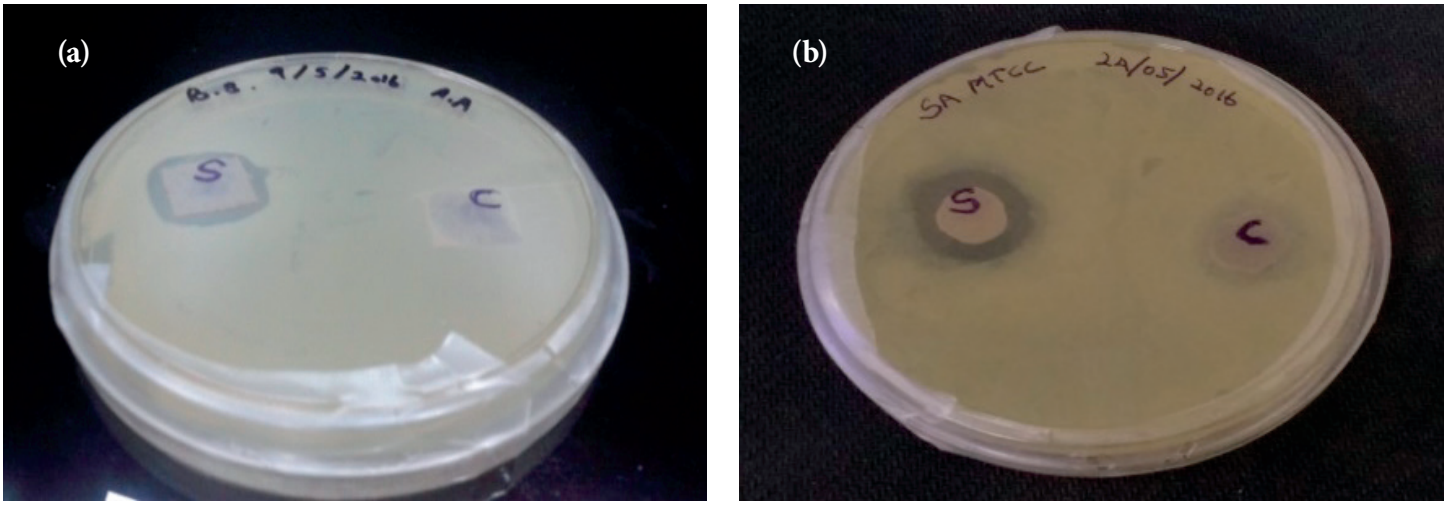

Figure 3. Photographic images of the zone of inhibition of Nanoparticles-BCS combination and tanned leather against **(a)** *Staphylococcus aureus* and **(b)** *Bacillus subtilis*

property of the combination tanning system compare to the conventional tanning process.

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Research Advances in Oil Tanning Technology: A Review

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Abstract

The conventional process of chamois leather manufacturing for industrial applications utilizes fish oil which contains substantial amounts of pentadienoic fatty acid. The applications of chamois leathers include cleaning polished surfaces, manufacture of gloves and orthopedic uses. However, due to fish oil's strong odor and high cost, considerable efforts have been made to counter these challenges. Esterification of fish oil has been used as a strategy to address the problem of odor and water absorption but this cannot solve the issue of cost. Oils from plant sources such as linseed, rubber, jatropha, castor and sunflower have been investigated as potential tanning substitutes for fish oil. Linseed has been found to produce chamois leather with mild odor and water absorption characteristics close to those of fish oil compared to other oils obtained from plant sources. Oil from goat fleshing has also been investigated in chamois leather production and has been found to produce chamois leather whose odor compares with that of linseed oil tanned leather. If these tanning oil alternatives are combined with other research advances in chamois leather production such as glutaraldehyde pre-tanning and oxidation using hydrogen peroxide or through ozonation, then the issue of cost, odor and long oxidation period can be resolved.

Introduction

Leather is one of the most widely traded commodities in the world, giving the leather industry an estimated global trade value of $414 billion per year.[1] It is generally processed in three steps, with the first step being pre-tanning. Pre-tanning involves soaking, liming and unhairing, deliming, bating, pickling and degreasing to remove unwanted components, hair, adipose tissue, fats, etc., leaving behind a network of fiber protein.[2] The next step, tanning, involves reacting the pre-tanned material with suitable agents to produce a stabilized fiber structure.[3] This can be achieved through chrome, vegetable, combined tanning or oil tannage. Finally, post-tanning - which involves neutralization, retanning, dyeing, fatliquoring and finishing in order to improve fiber characteristics and surface - is carried out. Finishing enhances color, softness, and lubrication of the leather surface.[4]

Tanning converts perishable organic material (animal skin) into a stable material (leather), which is resistant to spoilage by bacteria.[5] The skin is transformed into a non-putrescible matrix with increased hydrothermal stability via new cross-links that achieve additional dimensional stability within the matrix.[6] The most common inorganic tanning agents are chromium (III), zirconium (IV), aluminum (III), titanium and Iron.[7] Basic salts of these agents form coordinate bonds with the fiber structure and facilitates the stabilization of the protein structure.[8] Reactions take place at the carboxyl group of the collagen.[2] On the other hand, organic tanning agents are carbon and hydrogen containing compounds which have the ability to stabilize collagen within the skin. They include vegetable tannins, synthetic tanning agents (syntans), aldehydes and oils.[8] This review is focused on assessing the recent advances in oil tannage. These advances can be utilized by manufacturers to address challenges such as odor and cost faced by the chamois leather industry.

Applications of Oils and Fats in Leather Industry

Leather industry has found a wide range of applications for oils and fats. Rubber oil,[9] epoxidized oil,[10] and recovered olive oil[11] are examples of oils that have been used in oil tannage. Fatliquor-cum-filler production from fleshing waste for re-tanning has been studied.[12] The leather is fatliqoured to prevent fiber sticking when the leather is dried after completion of the wet processes.[13] Other studies on fatliquoring using seal hides oil, limed fleshing oil, synthetic, sulphited and sulphonated fatliqours have been reported.[14-17] In addition, oil has been reported as a potential formaldehyde scavenger.[18] The authors reported that use of 2% *Origanum onites* oil in the fatliquoring process substantially reduced the free formaldehyde formed in the process and offered a solution of potential importance to the leather industry.

Although the grammage of the garment leathers is directly proportional to the amount of fatliquoring agent, the drapability coefficient, stiffness and bending rigidity values also decreases with increase in amount of fatliquor.[11,19] Consequently, softness and drapability properties of garment leathers are greatly improved through fatliquoring. This indicates that leather manufacture can be influenced by the amount of oil used based on its application. Oil tannage yields low density and soft chamois leather, whereas fatliquoring is used to impart drapability in leathers tanned using other tanning technologies such as chrome tanning.

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However, it should be noted that other studies to assess the potential applications of such oils outside the leather industry have been carried out. Kolomaznik and co-workers experimented and modeled the potential of tannery fleshing waste as source of biodiesel.[20] The study proposed that oils from fleshing waste can be combined with oils from plants, such as used or waste cooking oils, for biodiesel production. Leather solid waste has also been investigated as a source of biodiesel.[21] The waste mainly originated from pre-fleshing, fleshing, shaving and trimmings, which were found to contain high amounts of fat contents. These studies point to a possibility of competition for tannery fleshing oils. Nonetheless, tannery fleshing waste is a serious challenge faced by the leather industry and any potential use for the waste will be highly welcome. Furthermore, an understanding of the various usages will spur more studies aimed at optimizing the applications of these oils.

Sources of Oils for Leather Industry

Chamois leather is popular in the leather market due to its unique properties. The process of chamois leather production conventionally relies on fish oil for its survival.[9] Attempts to come up with alternative sources of oil have been made, though their usage in the process is not adopted by the industry. Jatropha oil,[22] tannery fleshing oil,[23] linseed, castor and sunflower oils[24] have been experimented in chamois leather manufacture. Other applications such as fatliquoring that make use of seal-hide oil,[14] and oils from fleshing waste for re-tanning process[12] have been reported in literature. The modeling of fleshing oil production[20] and the practical extraction methods for these oils are also well documented.[25,26]

Chemical Characteristics of Tannage Oils

Studies on the chemical characterization of oils used in leather industry have been carried out and results are summarized in Table I below.[23,24] Iodine value is an indicator of the average degree of unsaturation (alkene group presence) within lipids while acid value shows the amount of free fatty acids present in fats or oils. Acid value is the amount of potassium hydroxide required to neutralize one gram of a fat or oil sample.[27] Saponification value indicates the average molecular weight of the triacylglycerol in a sample.

Acid values for the investigated oils average between 3 - 7 mg/g except for fish oil with a value of 15 mg/g. Despite most of the values being less than half that of fish oil, the studies have shown linseed and goat fleshing oils are promising oil tannage alternatives. However, the researchers did not provide details on animal tallow used and, therefore, the provided acidity value may not be compared to those of other oils. On the other hand, saponification values for all the oils are comparable, ranging between 174 - 188 mg/g.

Interestingly, among the characterized and investigated oils as substitutes to fish oil, linseed and goat oils are the best options for chamois leather manufacture on the basis of the organoleptic properties of the leathers produced (Table II). The organoleptic properties of the chamois leathers obtained using the two oils as tanning agents are of internationally acceptable standards.[30,31]

Characteristics of Oil Tanned Leathers

Various tests are carried out in order to grade chamois leathers. Water absorption, shrinkage temperature and organoleptic properties are some of the parameters that are used for this purpose. Table II compares the properties of leathers produced via oil tannage.[23,24]

Although not all data is available on the different oils for comparison purposes, it is useful to compare the available information. For example, linseed and goat oil produce chamois leathers with organoleptic properties that are comparable to those of fish oil except for odor. Water absorption and other features for the leathers obtained from the two oils are also within international standards.

A further comparison, based on the chemical characteristics of various oils presented in Table I and the properties of chamois leathers obtained from the oils (Table II) above, is also imperative. It is noteworthy that the top three oils (fish, linseed and goat) produce high quality chamois leathers. However, odor is rated poorly for fish oil-tanned chamois leathers. One outstanding disparity among the three oils is the acid value, which could be responsible for odor. Fish oil has acid value of 15 mg/g, way above the recommended value of less than 8.6 mg/g.[32] Conversion of fish oil into corresponding esters

Table I

Chemical characteristics of tannage oils

Parameter / Oil	Fish	Linseed	Castor	Sunflower	Animal tallow	Goat
Iodine value (mg/100 mg)	135	160	80	120	160	71
Acid value (mg/g)	15	7	4	6	3	7
Saponification value (mg/g)	180	188	176	188	174	186

Table II

Properties of oil-tanned leathers

Parameter	Fish oil	Linseed oil	Castor oil	Sunflower oil	Animal tallow	Goat oil
Water absorption	320 ± 5	303 ± 5	195 ± 5	197 ± 5	190 ± 5	211
Tongue tear	66.7 ± 0.5	55.0 ± 0.5	41.0 ± 0.5	39.0 ± 0.5	40.0 ± 0.5	-
Stitch tear	199.8 ± 0.5	154 ± 0.5	115.3 ± 0.5	109.3 ± 0.5	120.2 ± 0.5	-
Shrinkage temperature	83 ± 1	79 ± 1	70 ± 1	67 ± 1	72 ± 1	70
Sink test	120 ± 5	130 ± 5	155 ± 5	167 ± 5	158 ± 5	-
Softness	7	6	4	3	4	8
Fullness	7	7	5	4	5	-
General appearance	8	7	6	5	6	-
Odor	2	8	8	8	3	8

has been shown to help solve this problem.[33] Generally, esters are known to have sweet smell[34] and this may explain why esterification suppresses odor in fish oil tanned chamois leather. This may lead to the inference that acid value of an oil is inversely proportional to odor of chamois leather produced. However, more research needs to be done to validate this claim; as pointed out earlier, details of animal tallow used in Table II are not available and therefore the low odor rating (given as 3) cannot be used to conclusively establish a correlation.

Oil Tannage

Oil tannage is the method of making highly porous chamois leather.[8,32] The leathers are best known for properties of absorbing water, low density, softness and flexibility.[35] Chamois leathers are also highly resistant to the action of water at ordinary temperature.[36] They have found application in the manufacture of gloves, cleaning and drying of polished surfaces and in the production of orthopedic goods.[37] Different oils with tanning properties have been investigated for chamois leather production.[9,24,33] Characteristic tanning oil contains about 18 to 20 carbon atoms and about 4 to 8 unsaturated double bonds. The oil should have high iodine value of about 75 - 120 mg/g, a low acid value below 8.6 mg/g and it should not be rancid.[32]

The conventional oil-tanning process is based on the oxidation of cod, fish or sardine oils, which have numerous unsaturated bonds.[38] The unsaturated free fatty acids combine with oxygen to form oxidized form of fatty acids, aldehydes and peroxides which effect the tanning action on the pelt.[39] The level of unsaturation is imperative since little unsaturation in the oil will not oxidize promptly and, therefore, will work as an ointment whereas excessive unsaturation causes the oil to crosslink with itself and solidify upon oxidation. The tannage is alluded to an aldehydic reaction since the procedure is characterised by the release of acrolein, CH_2=CHCHO, and polymerization of the oil; the availability of latter could represent the variations between the qualities of oil and aldehyde-tanned leather.[32]

Traditional oil-tanning method

The traditional method of making chamois leather involved the impregnation of the skin with cod oil in fulling stock and then allow oxidation of oil to occur, with the products of the reaction having a tanning reaction.[9] This procedure is repeated until the point when pleasant leathering is accomplished. Surplus oil from the skin is expelled by pressure driven squeezing after washing in warm soluble water. The skin is then hung to dry and wrapped up.[40]

Modern oil-tanning method

The principle of modern oil tannage is to oxidize the fish oil, already introduced to the deemed pelt, with the help of atmospheric oxygen under controlled conditions. As the tanning agent, unsaturated glycerides like cod oil and fish oil are used.[41] These fatty acids having up to six double bonds in the aliphatic chain, give the necessary reaction products from oxidation and polymerization to give the characteristic chamois leathering effect under normal condition of tanning.[36]

Challenges and Advances in Oil Tannage

Time of oxidation and toxicity are some of the initial challenges that faced the manufacture of chamois leather. Traditionally, tanners used to impregnate skins or splits with fish oil and then left them to hang for up to 14 days to allow for air oxidation.[39] These long periods of oxidation made many tanners to shift to other methods of leather manufacture such as chrome tanning. However, mild pre-tanning operation using formaldehyde[42] in an attempt to address this issue

has since been abandoned due health reasons.[43] Glutaraldehyde has been espoused in its place. In addition, hydrogen peroxide, epoxidized oils, benzoyl peroxide or ozonation reactions have been studied as a way of accelerating the oil-tanning process.[39,42,44,45] These advances in oil tannage have reduced the time significantly to the point that oil tanning can now almost rival the other techniques of tanning.[23]

Cost and odor, especially for fish oil, are other factors that have hindered chamois leather manufacture. Concerted efforts have been made to address these problems. As already pointed out earlier, the use of linseed oil and oil from goat fleshing waste have been investigated and found to produce chamois leather that meets recommended standards. Nonetheless, linseed oil may face the challenge of cost due to its numerous uses.[46] Driven by competing demands for linseed oil, availability of the oil for tanning will not be assured. And even if it will be available, price will be driven by demand. However, oil from tannery fleshing waste can be an appealing alternative because its utilization will be a part of waste handling. Tanneries are currently grappling with fleshing waste management.[47-49] The waste produces foul smell and also piles up to the extent of minimizing available space within the tanneries or dumpsites. Thus, any alternative way of exploiting this waste will free the dumping space and yield additional income from the utilization of the extracted oil. Such value addition strategies will encourage adoption of this alternative source of oil. Countries such as India will greatly benefit from the advances in this field.[50] Availability of goat skins in India is very high and research has shown that these can be a source of fleshing waste for extraction of tanning oil.

Conclusion

With all the recent advances in oil tannage, it is possible to come up with an optimal combination of these innovations and produce chamois leather within reduced time and cost, and with desirable organoleptic and other physical features. Linseed oil and oil from tannery fleshing operations have been presented as potential alternatives to fish oil. However, linseed has competing applications and this might choke its supply. Coupled with glutaraldehyde pre-tanning and oxidizing agents such as hydrogen peroxide, these advances are likely to address the challenges in chamois leather manufacturing. More research needs to be carried out to actualize this at tannery level.

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Exploring DNA Diversity in Leathers: An Approach on Identification of Origin

by

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Abstract

DNA based approaches have become widespread in recent times to identify the origin of samples when its phenotypic characteristics are not distinguishable. This, in particular, applies to the leather industry wherein with an increase in duplicated embossing of grain patterns; there is a need to detect the animal origin of commercial leather articles. Thus, the characterization of molecular markers that enables rapid detection of the leather source helps us in precise species identification. The present study aims to generate definite sequences between the four major species in the Bovidae family (Buffalo, Cow, Goat, and Sheep), which are the major players in the manufacture of leather products, especially in India. Based on specific mitochondrial sequences, a specific fragment of the mitochondrial 12SrRNA gene was amplified by PCR as a marker for species-level identification. By the maximum homogeneity, from the NCBI and BOLD database, the BLAST analysis of the sequences of amplicons from unknown sources, distinguish closely related species of the subfamilies Bovinae (Buffalo and Cow) and Caprinae (sheep and goat) and this 12SrRNA based PCR-BLAST analysis is a good tool to identify the origin of leathers that are being manufactured. The present study has optimized an approach for the extraction and amplification of DNA from the finished leather, which is one of the most significant challenges because of the vigorous processes encountered during their manufacture. The findings of the study have commercial value at large scale.

Introduction

Leather, a highly commercial value product of animal skins or hides of the bovidae family subjected to various unit operations as shown in Figure 1. The process involves huge pH variations in order to facilitate the skin protein layers to be stabilized or tanned. Until tanning, the animal origin of the tanned material has been approximately identified based on the size and thickness of the skin or hide. Since the grain pattern of the skin or hide has significant variations, it has also been taken as a tool for the identification of the animal origin.[1] However, when the tanned material is transformed into a finished leather product, the loss in the surface as well as thickness pose challenges in the identification of animal origin. Further, to satisfy the need of the customer on design and pattern, and with the advancement of technologies, the original grain pattern of the skin/ hide has been masked with the required patterns through embossing, which completely affect the identification processes at significant level. Though other chemical and protein analyses have been suggested[2, 4] but it holds good with the untanned material and not for the finished leather, wherein, the protein has been highly stabilized with the tanning agent.

In order to have a reliable and highly precise methods to distinguish the animal species of the finished leather products, DNA based paleogenetic approach may provide the solution.[2, 5] The sustainable identification capability is possible with the combination of DNA sequence which provides an efficient and substantial path for species authentication.[6-8] With reference to the finished leather product, since, the leather making process itself eventually affects the survival of DNA (a wide pH variations in the process as detailed in the Figure 1), the DNA profiling of finished leather product[9, 10] is challenging. Similarly, Ojeda et. al.,[11] reported that it is difficult to discriminate the leather manufactured from Caviidae, Tayossuidae and Suidae families after the tanning process. Hence, the authors suggested the molecular approach, which solved the problems associated in identifying the animal origin of the products obtained from the said three families. In addition, the previous literatures have documented the survival of mitochondrial DNA (mt DNA)[12,24] in the 19th century leather articles, and thus provides a meager chance for the molecular traceability of the species in leather product samples and articles[13] and accordingly extracted the mtDNA from the finished leather samples of three families. A report on sheep and goat[2] employed only Chamois leather and a recent report[3] emphasizes the species identification using mtDNA with the amplicon size ranging from 52-75 bp. The results of the said studies may give a solution for the identification issues of leather samples of the bovidae family, however, still more authentication reports are in demand. Since, the finished leather products of the bovidae family have numerous chemicals which affect the extraction of the traceable quantity of mtDNA which in turn might also significantly affect the amplification process.[9]

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Leather from the bovidae family occupies the major place in the Indian leather sector, identification of the species from which t he leather has been made is vital in the current scenario to control the fraudulence happening. Hence, comparisons of nucleotide sequences of complete mtDNA or of various regions (for e.g., genes encoding cytochrome b,[14] cytochrome oxidase subunit-1 (COI), 12S rRNAs[15] and 16S rRNAs),[16] find helpful in understanding the phylogenetic relationships between species.[17-21]

Thus, the present study aims to develop a simple and reliable technique to discriminate the bovidae family animal origin of the finished leather product by following the refined DNA extraction procedures, construction of highly specific and sensitive primers, PCR amplification, sequencing o f amplified product an d authentication of the study with unknown samples. This method may prove to be an efficient method to control the quality of the leather manufactured and produced, reducing the occurrences of duplications.

Experimental

Sample collection

The finished leather samples for all the four species (Buffalo, Cow, Goat and Sheep), were collected from the Leather process Division of CSIR-Central Leather Research Institute, Adyar, Chennai, Tamil Nadu, India. All the finished leather sample products were tanned with basic chromium sulphate. The thickness/grain-pattern of all the test samples were measured/observed for morphological validation of the species before further investigation.

DNA extraction from leather samples

The collected finished leather product samples were finely chopped to small pieces using sterile scissors and blades. DNA was extracted from leather samples according to the procedure described[11, 22, 23] with few modifications to have better DNA yield. The optimisations to the final DNA extraction method was done after several trial and error runs. The known finished leather samples of 25 mg was placed in a 1.5-mL tube and 500 µL lysis buffer (10-150 m M Tris-HCl, pH 8.0 – 9.0, 10 - 150 mM EDTA, 2.0-3.0% SDS, and 10- 150 mM NaCl) and 20 µL proteinase K (20 mg/mL, GeNei) and 5 µL of Rnase A (10 mg/ml, Thermo scientific™) was added and the samples were incubated at 50°-60°C in a dry bath for 8 - 12 h with pulse stirring. The general protocols such as centrifugation and t he supernatant collection done accordingly to separate the DNA. One µl of the obtained DNA was used for determining DNA concentration and quality in Biotek micro plate reader. The samples were then subjected to electrophoresis on a 0.7% agarose gel in 1X TBE and the presence of DNA was quantified with a gel dock (Medicare Gelstan).

Primer Design

Table II describes the primers specific to the region corresponding to mitochondrial 12S rRNA obtained from *in silico*. All the published sequences of the four animals were collected from NCBI genbank and performed the multiple sequence alignment using Clustal W (EMBL/EBI). Primers were designed for each Genus according to the conserved region within the genus and hypervariable between genus. Although there were many priming sites, the primers that correspond to in silico amplification and no cross reactivity of all the four selected sequences were selected. The designed specific target gene primers were synthesized (Eurofins Genomics India Pvt Ltd). Further, to ensure the specificity of the designed primers, we amplified the primers on the freshly extracted DNA. The amplicons were extracted, sequenced and analyzed using NCBI Blastn database. The sequencing results confirmed the specificity of the designed primers by only amplifying the respective target gene of the corresponding animals and does not have a cross reactivity.

PCR Amplification of Control and Test samples

Thermal Cycling was conducted with 1U of Taq DNA polymerase in a 25-µl reaction mixture, consisting of 50 ng of genomic DNA, 1.5 µM of each primer (Eurofins Genomics India Pvt Ltd), 200 µM deoxyribonucleotide triphosphates (dNTPs) and 1X Taq buffer (Bangalore Genei, India) with 1.5mM Mgcl2. With the prepared template and primers, PCR was conducted using the following temperature profile: initial denaturation at 95°C for 3 min, then 35 cycles of 45 Sec at 94°C, 45 Sec at 54.3°C, and 45 Sec at 72°C; and finally, an extension reaction of 3 min at 72°C. The eppendorf Thermal cycler (Eppendorf North America Inc) was used for PCR reaction. In the present study, the normal Taq polymerase was used whereas the hot start Taq polymerase may be used in future experiments for the betterments in the amplification specificity.

For test runs, both the quantity of DNA and the cycle runs were varied to have the optimum conditions. DNA quantity ranging from 1 to 100 ng and the cycle numbers were of 30, 35, 40, 45. Every sample was amplified with respective primers twice to check the reproducibility. The amplified products were electrophoresed with DNA ladder (Step UpTM 100bp DNA Ladder, Bangalore Genei, India) on 2 % agarose gel. The amplified PCR bands were subsequently visualized in UV transilluminator for the amplified fragment size validation (Gelstan 1612, India).

DNA sequencing

The PCR products were eluted and purified using QIAquick Gel Extraction Kit (Qiagen, Inc. Valencia, CA, USA). Sequencing was performed on 3500 Genetic Analyzer, Applied Biosystems (CA, USA) using the Big DyeTM terminator cycle sequencing ready reaction mixture in accordance with manufacturer's instructions. The sequences retrieved were analyzed by sequencing analysis software 6 v6.0, Applied Biosystems (CA, USA).

The expected amplified DNA band for all the test samples were excised from gel and purified using the QIAquick Gel Extraction

Kit (Qiagen, Inc. Valencia, CA, USA) as per the manufacturer's protocol. Sequencing reactions were carried out with a BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) using the PCR-primers as sequencing primers by using ABI Prism 3500 genetic analyzer (Applied Biosystems, Foster City, CA, USA). The sequences thus obtained were assembled and edited using Sequencing analysis software 6 v6.0, Applied Biosystems (CA, USA).

Authentication of the method

In order to further authenticate the protocol and the primers designed for the Bovidae family, a double-blind study was conducted using unknown (test) finished leather samples received from the tanning industries. Further, a skin sample received from aquatic species has been considered as a negative reference.

Bioinformatics Analysis

Identification using BLAST

Obtained sequences were run for BLAST search against public database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?LINK_LOC=blasthome&PAGE_TYPE=BlastSearch&PROGRAM=blastn) to determine the closest match of unknown specimens.)

Results and Discussion

Morphological analysis

Figure 1a illustrates the steps involved in the leather manufacturing process from animal skin/hide of the bovidae family along with the morphology of the samples obtained from each step for a representative animal skin. Figure 2a illustrates the representative

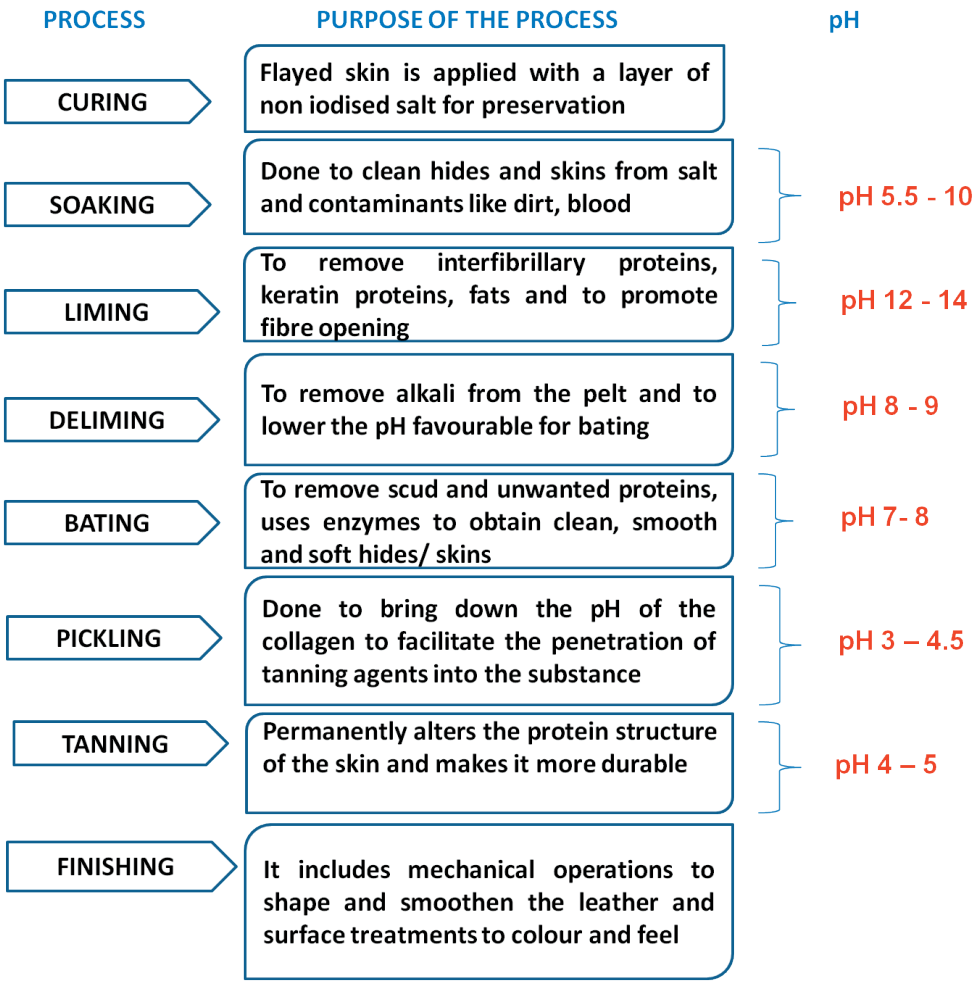


Figure 1. Schematic representation of unit operations of leather processing wherein the animal skin/hide has been transformed to finished leather. The pH variations in each unit operations were described in the figure. In brief, during the leather production, the skins and hides are subjected to various pH conditions which plays a significant role in the stability of the nucleic acids. These pH conditions lead to the degradation of the DNA present in the skin/hide thereby the survival rate of nucleic acids are very negligible. Therefore, to increase the rate of success in PCR, mtDNA was used as a template in the amplification in the present study. As the copy number of the mtDNA was higher than that of the autosomal DNA (Each cell contains 2 copies of nuclear DNA while the same cell contains 100 - 10000 mitochondrial genomes) mitochondrial DNA is preferred as template in PCR.

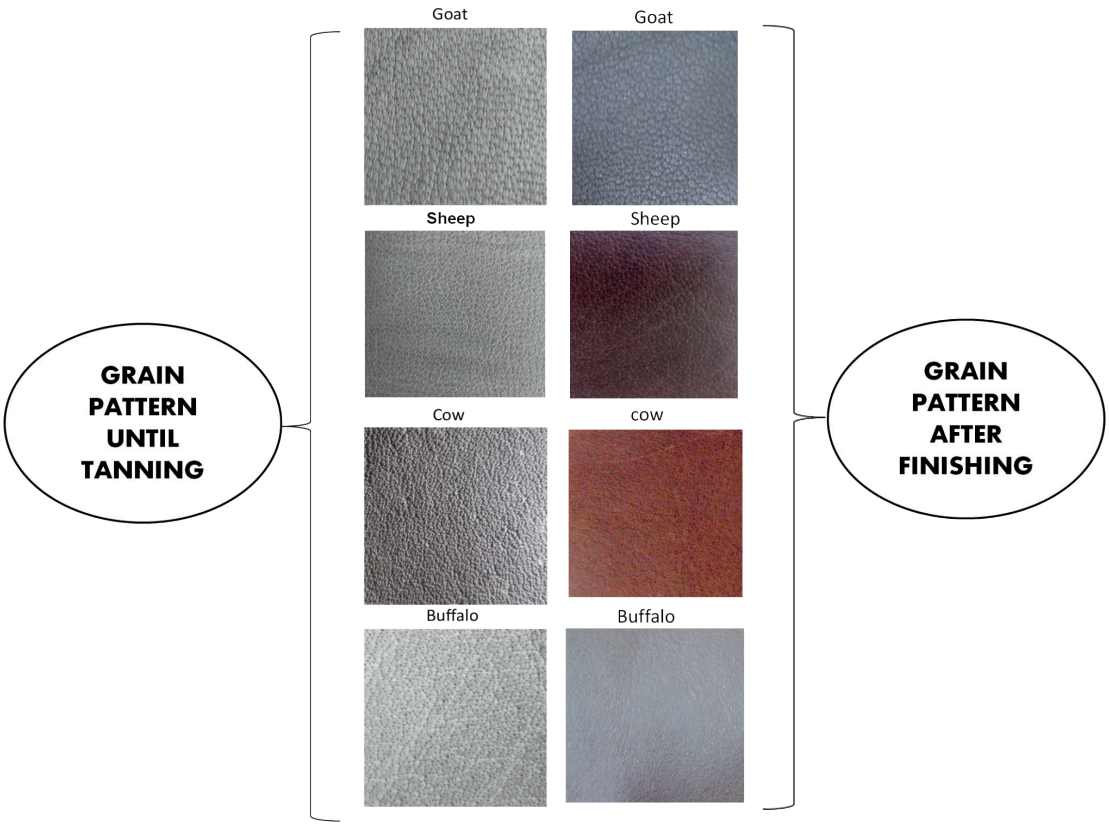
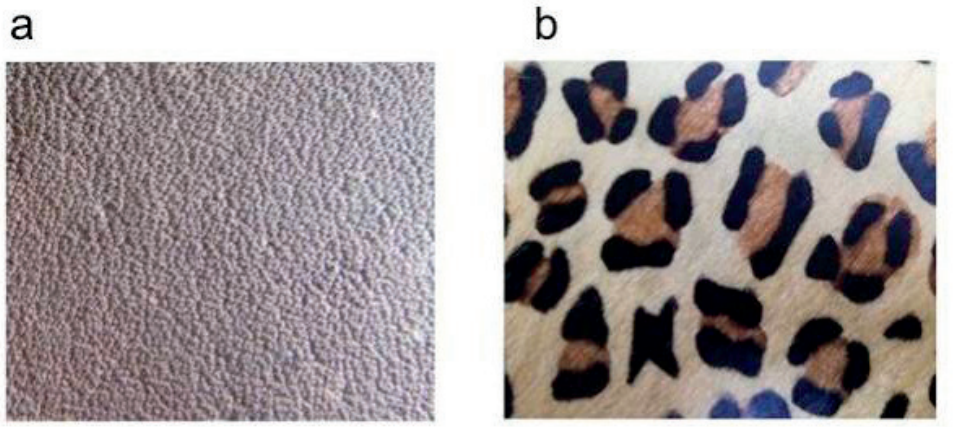


Figure 2a. Representative image on the comparison of Grain patterns of the leather samples of bovidae family until tanning and after finishing. The image indicates the changes in the morphological characteristics of the grain pattern present in the leather during pre-tanning and post -tanning operations in leather making process.



(a) - Cow finished leather (hair free tanning process) grain pattern – optical view
(b) - Cow finished leather (hair on tanning process) with leopard pattern

Figure 2b. A representative image on the embossing of leopard pattern on the hair on tanning finished cow leather. The image depicts an illustration on how a common end user cannot distinguish the cow leather and a leopard leather. Visual inspection may not correctly identify the species of leather at every time, hence, further authentication involving molecular techniques is a necessity.

Table I

Comparison on thickness profile of the animal skin of the Bovidae family until tanning and after finishing

S. NO	Species	Thickness of the material (mm)	
		Until tanning	After finishing*
1	Cow (Calf/Hide)	1-6	0.9 -3
2	Buffalo (Calf/hide)	1-8	1-3.5
3	Sheep skin	0.5-2	0.5-1.5
4	Goat skin	0.5-2	0.5-1.5

(*Finishing involves, shaving, splitting, dyeing, fatliquouring, setting, staking, buffing and toggling. Splitting of leathers for the requisite purposes may have different thicknesses).

image on the grain pattern of skin/hide samples before and after finishing. Figure 2b illustrates the embossing of leopard pattern on the cow finished leather, wherein the process of cow skin/hide involves hair on tanning. Table I depicts the thickness profile of the animal skins of the bovidae family untill tanning and after finishing.

As described, the finished leather samples have different thickness and have embossed patterns and have been considered as the major challenges in the identification of animal origin. The rigorous manufacturing steps as described in Figure 1 suggests the wide pH variations in each step. During the first step, the skin/hide samples have been exposed to high alkaline pH which support the removal of the hair and flesh in the presence of reducing agents and then exposed to acidic pH which supports the tanning process and again brought down the pH to near neutral during the post tanning operations which includes, dyeing, fatliquoring, etc. for the better quality and organoleptic properties. The finished leather samples thus obtained may or may not have macromolecules like DNA and if at all may be in very meager quantity. Hence, the prime task of the present study is to recover the low abundance of mtDNA and also to maintain the quality of DNA from the finished leather samples of the bovidae family by cost effective methods. The extraction process does not involve phenol –chloroform or guanidinium thiocyanate/ silica. But, the process employs modified protocol of Ojeda et al.[11]

DNA extraction from finished leather samples

The DNA extracted from the known animal origin of finished leather products of the bovidae family suggested the reliability and the precision of the procedure followed in the present study. The method yielded plenty of good quality of DNA required for the present study.

PCR amplification and DNA sequencing of control and test samples

Table II describes the species specific primers to the region corresponding to mitochondrial 12S rRNA obtained from *in silico*. All the animals included in the study belong to the Bovidae family, so designing a species specific primer set with short amplicon size was not possible as they are closely related. Figure 3 (a-d) depicts the PCR amplification profile of four finished leather samples chosen for the present study. The amplicon size for respective animals were identified as 331 bp (Goat), 356 bp (Sheep), 280 bp (Cow) and 451 bp (Buffalo) which matches with the primers as designed inaccordingly.

Following the assessment on known leather samples, experiments were conducted with the unknown samples (10 numbers) received from tanning industries in Tamil Nadu, India, which includes leather from aquatic animals (Fish). These test samples upon extraction of DNA as per the modified procedure described in the present study were then amplified with the designed primers. Figure 4 (a-d) depicts the PCR amplification profile of the test samples. It has been observed that in Figure 4a, the amplicons of the two samples among the test samples matches with the goat (331 bp), whereas in Figure 4b, the amplicons of the three samples fit with sheep (356 bp). Figure 4c represents the two amplicons that matches with the cow (280 bp) among the unknown samples and the Figure 4d, illustrates three samples that resembles buffalo (451 bp). The last lane displayed in all the gel results represent the negative control.

Table II

Details on primers of specific region chosen for the animals of bovidae family for identification

Primer ID*	Sequence	Basepair length	Target
Goat	5' - AGCCCGAAACTCAAAGGACT - 3'	331	12S rRNA mtDNA
	5' - CGTGCTTCATGGCCTAATTC - 3'		
Sheep	5' - AGCCCGAAACTCAAAGGACT - 3'	356	12S rRNA mtDNA
	5' - CGTGCTTCATGGCCTAATTC - 3'		
Cow	5' - ACCGCGGTCATACGATTAAC - 3'	280	12S rRNA mtDNA
	5' - CGCCAAGTCCTTTGAGTTTT - 3'		
Buffalo	5' - CAAACTGGGATTAGATACCCCACTAT- 3'	451	12S rRNA mtDNA
	5' - AGGGTGACGGGCGGTGTGT - 3'		

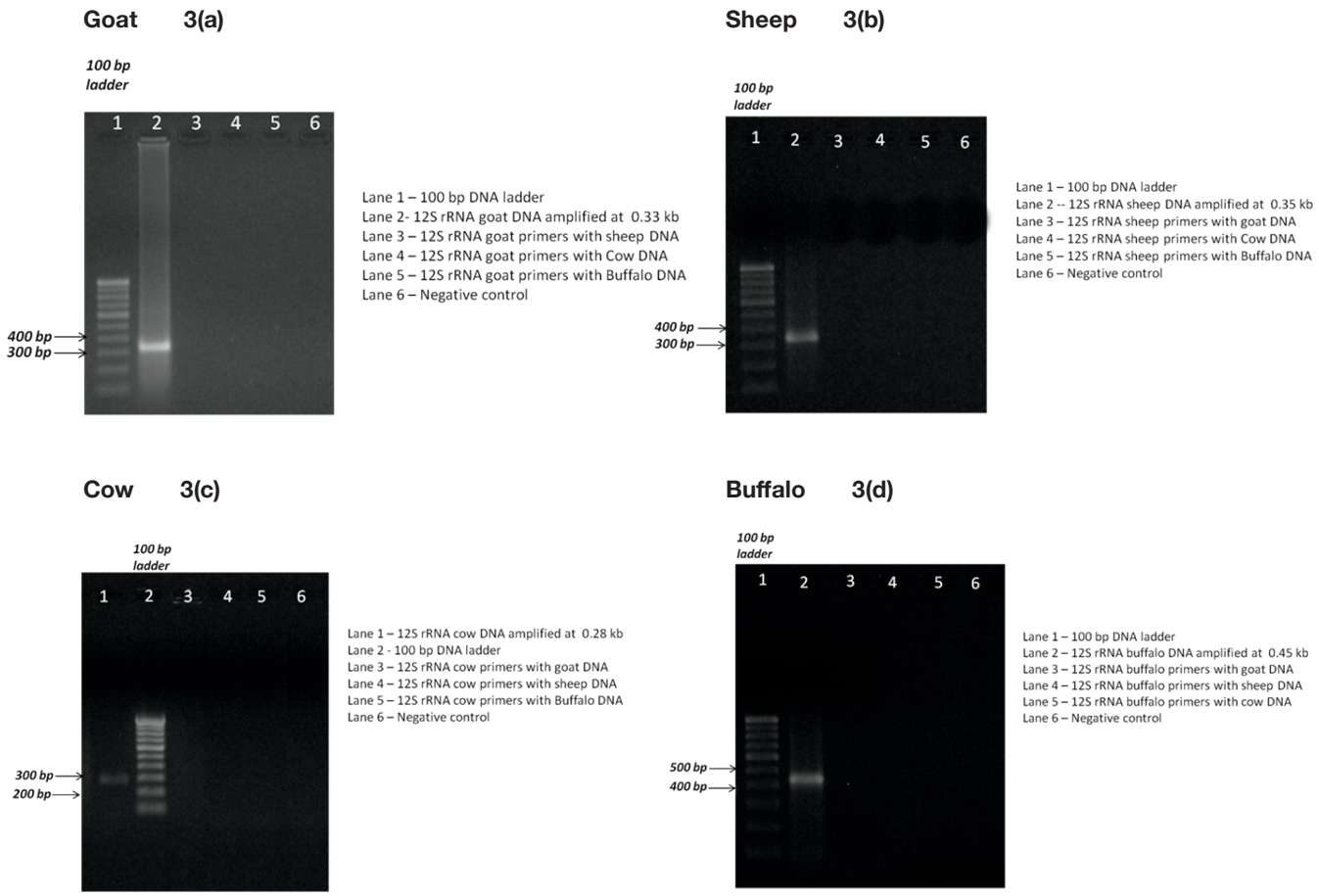


Figure 3 (a-d). PCR amplification profile of the four finished leather samples of Bovidae family, indicating amplification of Goat, Sheep, Cow and buffalo at respective basepairs, viz., 0.33 kb, 0.35 kb, 0.28 kb, 0.45 kb.

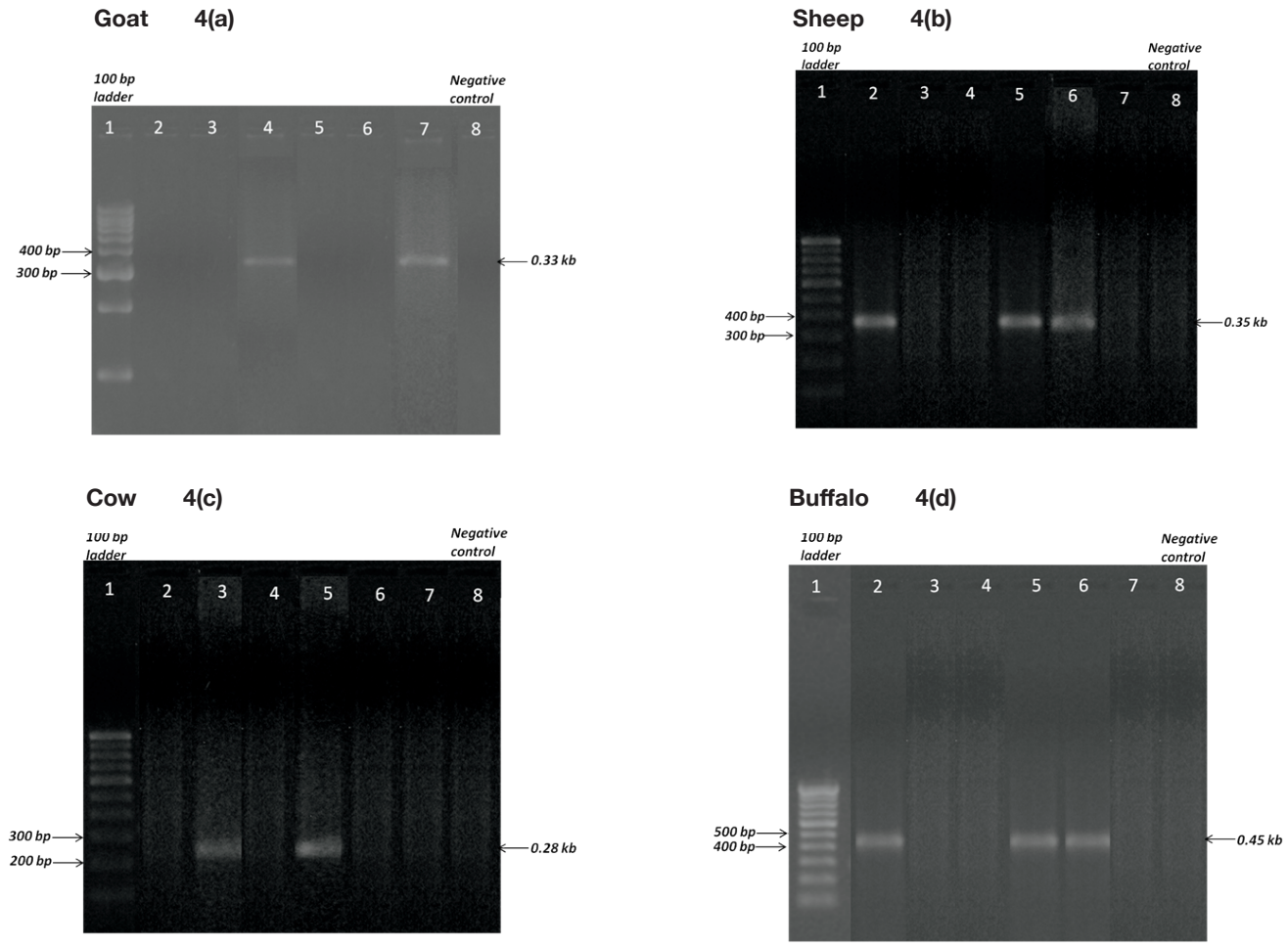


Figure 4 (a-d). PCR amplification profile of the test (unknown) samples. In fig 4a, the amplicons of the two samples matches with the Goat (331bp) while in fig 4b, the amplicons of the three samples matches with sheep (356bp). The fig 4c, represents the two amplicons that matches with the cow (280 bp) and the fig 4d, illustrates three amplicons that resembles Buffalo (451 bp). The lane 8 in all the four animals represent the negative control.

The present study on identifying the animal origin of the finished leather samples of bovidae family of Indian origin, suggested that with less survival of macromolecules (DNA), it is possible to extract the mtDNA from the finished leather samples using the modified method. Results on PCR amplification studies on known (control) and unknown (test) samples along with a negative reference sample (no template) evidently proved that the primers designed based on the conserved sequences are specific to the species and are able to amplify the target gene without any cross-reactivity. DNA based species identification does not require haplotype sharing. Moreover, this problem did not occur with 12S rRNA gene as its evolutionary rate is higher. The success rate of the statistical identification of species is high up to 99% with respect to 12S rRNA. The variable priming sites of 12S rRNA could be a great tool for identifying the source of an unknown leather sample, which comes under the Bovidae family, by BLAST analysis.

Sequence Submission to Genbank Database

The sequences retrieved were directly submitted to NCBI Genbank Database and received the Accession numbers as KX824125, KX824126, KX824127, KX824128.

Conclusions

In the present study, we have shown that the unknown (test) leather samples can be identified for its origin using the designed species specific 12S rRNA primers of the bovidae family. Also challenges faced during the isolation of DNA from leather samples were also discussed. Indeed, results of the present study have proved that the 12S rRNA based PCR- BLAST is one of the simplest and easiest methods for the identification of the leather sample origin and also helps in finding the genuiness among the leather samples.

Conflicts of interest

There are no conflicts to declare

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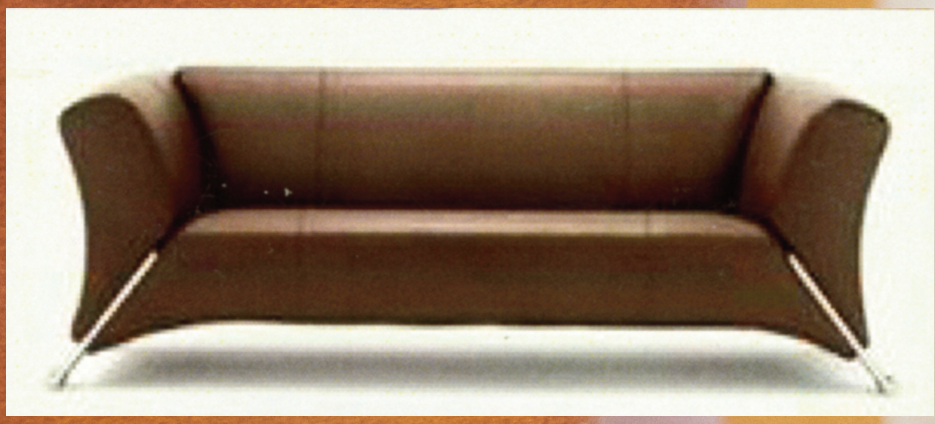

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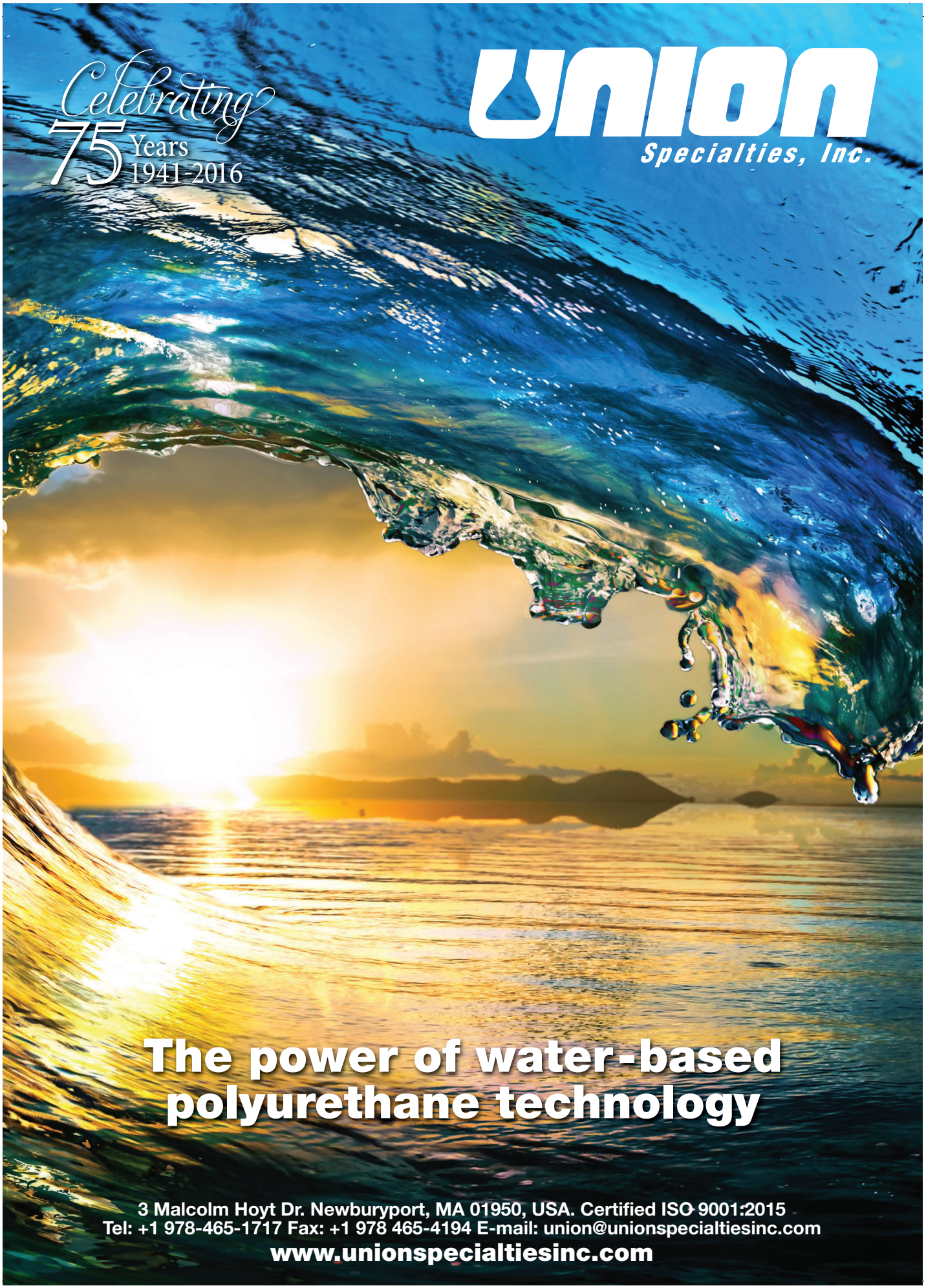
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Lifelines

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1906–1909; 1911; 1913; 1915; 1921
1937 – January and December
1939 – January, March and April
1940 – January

Extremely Low Supply:

1931 – February
1936 – January and February
1944 – Jan., Oct., Nov. and Dec.
1945 – June
1946 – January – April
1947 – January, April and August
1948 – January – March
1949 – January – June
1955 – January
1969 – March
1970 – February
1979 – July
1983 – March
1984 – July
1999 – June and July
2000 – January and February
2001 – May and June
2002 – January and March

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CALL FOR PAPERS

FOR THE 116th ANNUAL CONVENTION OF THE AMERICAN LEATHER CHEMISTS ASSOCIATION

Eaglewood Resort & Spa, Itasca, Illinois
May 4-7, 2021

If you have recently completed or will shortly be completing research studies relevant to hide preservation, hide and leather defects, leather manufacturing technology, new product development, tannery equipment development, leather properties and specifications, tannery environmental management, or other related subjects, you are encouraged to present the results of this research at the next annual convention of the Association to be held at the Eaglewood Resort & Spa, Itasca, Illinois, May 4-7, 2021.

Abstracts are due by February 1, 2021
Full Presentations are due by May 1, 2021

They are to be submitted by e-mail to the
ALCA Vice-President and Chair of the Technical Program:

Joseph Hoefler
The Dow Chemical Company
400 Arcola Rd.
Collegeville, PA 19426
E-mail: jhoefler@dow.com

The **Abstract** should begin with the title in capital letters, followed by the authors' names. An asterisk should denote the name of the speaker, and contact information should be provided that includes an e-mail address. The abstract should be no longer than 300 English words, and in the Microsoft Word format.

Full Presentations at the convention will be limited to 25 minutes. In accordance with the Association Bylaws, all presentations are considered for publication by *The Journal of the American Leather Chemists Association.* They are not to be published elsewhere, other than in abstract form, without permission of the *Journal* Editor. For further paper preparation guidelines please refer to the *JALCA* Publication Policy on our website: leatherchemists.org

Full Presentations are to be submitted by e-mail to the *JALCA* editor:

Steven D. Lange, *Journal* Editor
The American Leather Chemists Association
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Mobile Phone (814) 414-5689

116th ALCA ANNUAL CONVENTION Change of Date: May 4-7, 2021 Eaglewood Resort & Spa Itasca, IL

Featuring the 61st John Arthur Wilson Memorial Lecture
By Randy Johnson, President and CEO
of GST AutoLeather
Title: Road Ahead

Tentative Schedule

Tuesday, May 4
Golf Tournament, Opening Reception and Dinner

Wednesday, May 5
John Arthur Wilson Memorial Lecture
All Day Technical Sessions, Fun Run
Reception and Dinner, Activities - Bowling, Pool,
Darts and an Open Bar

Thursday, May 6
All Day Technical Sessions, Annual Business Meeting
Activities Awards Luncheon
Social Hour, ALCA Awards Banquet

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