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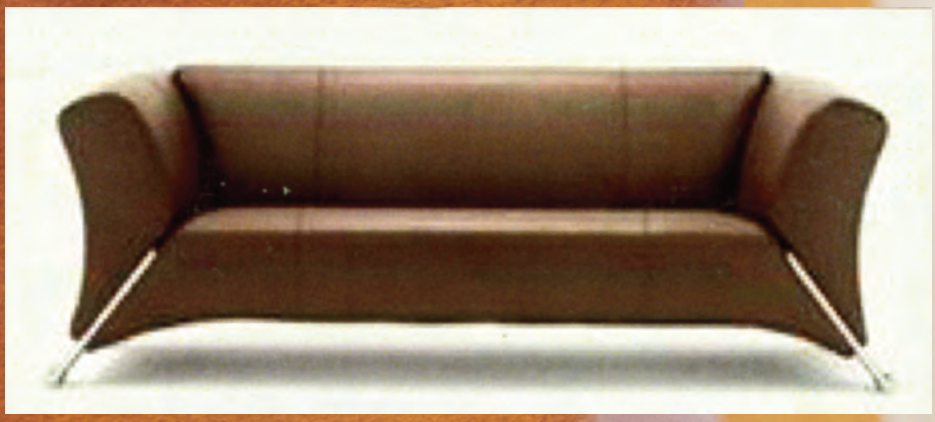

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Identification and Characterization of Potential Biocide-Resistant Fungal Strains from Infested Leathers – A Systematic Study

by
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Abstract

This study is aimed at identification of biocide tolerant/resistant fungal strains afflicting the leather industry. Fungal infestation occurs sometimes despite biocide treatment during leather processing. This persistent growth can be due to the development of biocide resistance which can lead to health hazards and economic loss. As no study has so far been reported to either confirm this or to identify such fungal strains, a systematic approach has been made in this study to address these aspects. Fungal strains were collected from infested leathers from tanneries to identify biocide resistant fungal strains afflicting leather industry. Phenotypic characterization revealed *Aspergillus* as the most dominant with 58% occurrence. Ten isolates were subjected to 18s rRNA sequencing and four strains were identified as *Aspergillus niger*. An *in-vitro* susceptibility to four leather fungicides was assessed to identify the biocide tolerant strains. S-6 *A. niger* strain was found to be the most tolerant as evidenced by high MIC (7.81µg ml^{-1}) against the most effective biocide, 2-(thiocyanomethylthio) benzothiazole. I*n-vivo* studies on chrome-tanned leathers also confirmed this finding. SEM studies revealed considerable morphological changes in S-6 compared to wild strain providing further evidence that it may have developed biocide resistance.

Introduction

Biocides are widely used in industries like agriculture, wood, leather etc., where the materials are prone to fungal attack.[1] The occurrence of fungal infestation on leather is a common problem, especially in conditions with high humidity.[2] Some of the fungi that are encountered in leather industry predominantly belong to genus of *Aspergillus, Penicillium, Rhizopus* and *Paecilomyces*. [3-6] The growth of fungi is controlled to a large extent with the aid of biocides. However, complaints regarding fungal growth exist despite the use of biocides during leather processing.[7] This might be due to improper use of biocides which includes insufficient dosage and concentration that may lead to poor distribution and uptake by the leathers. Insufficient contact time and inconsistency in biocide formulation can also result in reduced protection. Additionally, the most serious concern is with the probable development of biocidal resistance or tolerance in fungi. The possibility of the biocides loosing biocidal efficacy due to development of anti-microbial resistance (AMR) in some fungal species would present a difficult challenge.[8]

The infestation on leathers not only lessens the durability of the product but also decreases their commercial value.[5, 9] Fungal growth on finished leathers and products can lead to huge economic loss to the tanners as well as the buyers since remediation can be quite difficult. Hence, industries like leather sector needs to gear up to meet the emerging challenges on this front.

Resistance development towards antibiotics is well-studied and better understood than that against industrial biocides.[10] The mechanism of action pertaining to industrial biocides is also not well defined. The information available in the area of antibiotics is usually extrapolated to understand the biocidal mode of action.[11-13] Even though the concept of biocide resistance has been discussed in the leather industry, no systematic study is available either to confirm this or to identify the resistant strains of fungi.

Serious concerns regarding resistance development against biocides are expressed by various stakeholders of leather sector but the apprehension is mostly limited to protection against fungal infestation to prevent heavy economic loss. The tanners try to circumvent the problem by using biocides in rotation as well as employing new biocides in processing. But the AMR leading to serious health hazards due to possible cross resistance against antibiotics will have far reaching implications.[14, 15] Though, some chemical supply houses reject the idea of development of AMR in leather-borne fungi by arguing that it is a myth more than a fact,[16] the challenge on the front is imminent since, AMR development in clinical settings and in other areas have been proven to be real.[17] Concerted scientific efforts and studies are needed to understand the phenomenon and the mode of development in order to devise strategies for preventing the same.

Therefore, the present investigation focuses on isolation, screening and identification of biocide resistant strains. All the fungal isolates infecting leathers despite biocide treatment were collected from

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various tanneries and phenotypically identified. Few strains were selected for genotypic characterization and *in vitro* antifungal susceptibility test. An *in vivo* study on biocide treated leathers was also conducted to test the growth of resistant strain. As there is no well documented study in this aspect about the leather industrial biocides, measures were taken for a systematic study in identification of a dominant fungal strain. The selected strain can be further studied in detail to understand the mode of biocide action and the possible mechanism involved in the rise of biocide resistance.

Materials and Methods

Source of media and chemicals

Media and other chemicals were purchased from Hi-Media, India. Biocide formulations based on 2-(thiocyanomethylthio)benzothiazole (TCMTB), 2-Mercaptobenzothioazole(2-MBT), 3-iodo-2-propynyl-N-butylcarbamate (IPBC) and 2,2-dibromo-3-nitrilopropionamide (DBNP) were obtained from reputed leather chemical supply houses. Wild strain *Aspergillus niger* (ATCC 6275) used in this study was procured from American Type Culture Collection (ATCC).

Collection of fungal infected leather samples from tanneries

An initiative has been taken in this study to screen and isolate fungi which infect leathers despite biocide treatment. Tanneries often facing the problem of fungal infestation were identified and a total of six tanneries were selected in the state of Tamil Nadu (Figure 1).

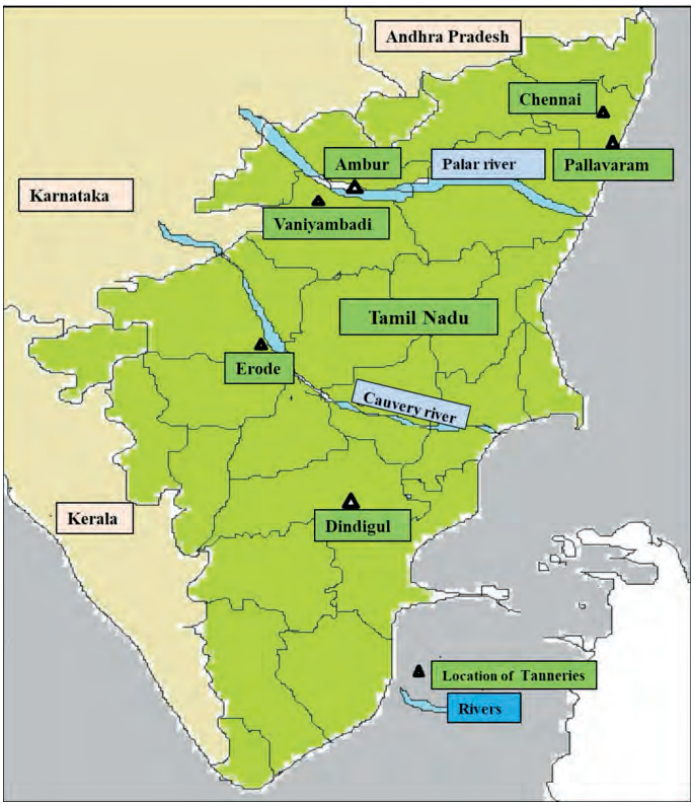

Figure 1. A map of State of Tamil Nadu, India showing the geographical location of tanneries for sample collection

Fungal strains were isolated from infested leathers, where the growth is prominently observed despite the biocide treatment. Fungal infested leathers (Figure 2) were sampled at different sampling sites within the tannery and were subsequently labelled. For example, in tannery 1, three different sampling sites were noted, and the affected leathers were collected, which resulted in a total of eight isolates. The same was followed in other tanneries and number of sites and the samples collected from each of them is presented in Table I.

A total of 35 fungal strains were obtained from leathers at different stages of leather processing, which were affected despite the biocide

Figure 2. Fungal infested leather samples collected from various tanneries

Table I

Sampling and Screening of fungal Isolates from various tanneries

Tannery Identified	Sampling sites	No. Fungi isolated
Tannery 1	Site 1	3
	Site 2	2
	Site 3	3
Tannery 2	Site 4	3
	Site 5	2
Tannery 3	Site 6	3
Tannery 4	Site 7	3
	Site 8	2
	Site 9	3
Tannery 5	Site 10	2
	Site 11	2
	Site 12	1
	Site 13	2
Tannery 6	Site 14	2
	Site 15	2

treatment. The infested biocide treated leather samples viz., chrome tanned, vegetable tanned, finished leathers and leather products collected around the year from different tanneries were brought to the laboratory for further investigation. Infested areas of leathers were cut using sterile blade and all the samples were labelled individually and placed in sterile zip-lock bags. These samples were examined microscopically for the fungal growth.

Isolation and maintenance of fungal cultures

The samples confirmed for the presence of fungal growth by microscopic examination were further subjected to isolation procedure. The spores present on the surface of the infected leathers were scraped using a sterile scalpel and placed on Sabouraud Dextrose Agar (SDA) plates and incubated *in vivo* at 30± 2°C for 5 to 7 d until sufficient growth was observed. Different fungal colonies observed on the plate were further purified on SDA plates and stored at 4°C.

Phenotypic characterization

The phenotypic identification of the fungal isolates was carried out by examining the macro-morphological features by colony morphology and growth characteristics of the pure fungal cultures as described by Raper and Fennel.[18] The features like colony diameter, mycelium color, soluble pigments and presence or absence of sclerotia were assessed.[19] Additionally, the presence or absence of fruiting bodies was also examined by visual and micro morphological observation.[20-22] The isolates were further examined by wet mount technique (Lobomed, Lx-300 microscope) using Lacto Phenol Cotton Blue (LPCB) as described by James and Natalie.[23] The spore size of conidia, conidiophores, and their arrangements were assessed by traditional method.[24]

Genotypic identification and Phylogenetic analysis

After phenotypic characterization, based on the occurrence and dominance of the fungal strains on leather, ten isolates comprising seven *Aspergillus* species with the labels S-1, S-4, S-5, S-6, S-8, S-9 and S-10, one *Rhizopus* sp. (S-3), one *Penicillium* sp. (S-11) and one *Paceliomyces* sp. (S-14) were characterized genotypically. The fungal cells obtained from the isolates grown in Sabouraud Dextrose Broth (SDB) for 5 d at 30± 2°C in an incubator shaker at 125 rpm were subjected to genomic DNA extraction. The fungal biomass in each case was harvested by centrifugation at 4000 × g for 5 min and washed twice with nuclease-free water. The DNA was extracted using HiPurA fungal DNA purification kit (Hi-Media, India) as per the manufactures' protocol.

The resulting PCR amplicons were purified and sequenced at Xcelris Genomics, India, using ABI 3730 × 1 Genetic Analyser, India. The obtained nucleotide sequence data was examined using aligner software to get consensus sequence which was further subjected to BLAST analysis using Blastn site at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST). Phylogenetic tree was constructed for the obtained consensus sequence using the software MEGA version 7.0 for each isolate.[25] The multiple sequence alignment was done using CLASTAL W programme[26] and neighbour joining method was adapted for tree construction.[27]

***In vitro* susceptibility of the fungal strains against selected biocides**

The minimum inhibitory concentration (MIC) was determined *in vitro* against the selected test strains by macrobroth dilution method following EUCAST methodology used for filamentous fungi (M38-A).[28] For testing the susceptibility of isolated fungi towards selected biocides, a stock solution (1 mg ml^{-1}) was prepared for all four selected biocides in sterile broth. The spore suspension (2-5x106 spores ml^{-1}) was prepared from fresh 5 d old fungal culture for inoculum.[29] Briefly, a set of 12 vials was taken for serial dilution and to each of them; 2 ml of sterile SDB was added. 2 ml of test biocide from stock of 1 mg ml^{-1} was added to the first vial and serially diluted till 10th vial to get the concentrations in the range of 0.5-1000 µg ml^{-1}. The 11th and 12th vials served as growth control and sterile control respectively. To each vial (1-11) except the sterile control, 100 µl of spore suspension was added. All the vials were incubated at 30± 2°C for 48-72 h to observe for the visible growth. The lowest concentration that completely inhibited the growth was determined as the MIC.[30] All susceptibility tests were conducted in duplicates.

Studies on the growth of resistant strains on biocide treated leather

The growth of most resistant strains of *A. niger*, S-1, S-4, S-6 and S-8, as evidenced from high MIC needed to inhibit them in the study described in the previous section, was studied in comparison with wild type ATCC 6275 on TCMTB treated leathers. The trials were conducted using pickled cow hide which was cut into pieces (18 × 10 cm), labelled and weighed. After this, the hide pieces were chrome tanned and treated with TCMTB at varying concentrations of 0.05%, 0.1%, 0.2%, 0.75% on the weight of pickled pelt. One piece served as control which was neither treated with biocide nor inoculated with any of the strains. The hide pieces were treated with biocide in a steel drum that runs at an rpm of 10 for 60 min for proper distribution of the biocide into the leathers. After this, the samples were removed from the drum and left for ageing for 48 h, maintaining them in moist condition. These test pieces were eventually subjected to tropical chamber evaluation by standard accelerated method (IS: 6191-1971) to determine the growth of the each of the fungal strains. For this, spore suspensions were prepared for all the fungi to be tested. The test leather samples were placed on petri plates and about 0.1 ml of spore suspension was smeared on the surface of the leathers. The samples were placed in a humidity chamber maintained at 30±2°C and 95-100% relative humidity and visually examined every day for the appearance of growth.

Scanning electron microscopy (SEM) analysis

SEM analysis was performed to observe morphological changes, if any, in *A. niger* test strains S-1, S-4, S-6, S-8 in comparison with wild type *A. niger*. Pure cultures of all five isolates grown for 5 d in SDB were harvested to obtain the mycelial mat. The obtained mycelial mat samples were fixed in buffered 3% glutaraldehyde (0.05M phosphate buffer, pH 6.5) and incubated at 4°C for 24 h. The

samples were then washed twice with sterile water and subjected to dehydration in ethanol series (10% – 90%) for 10 min each followed by two washings in 100% ethanol. The samples were dried by transferring them to critical point drier in the presence of liquid CO_2 and stored in a desiccator for further use. The specimens were mounted on aluminum stubs using suitable carbon tape and gold coated using an Edwards E-306 sputter coater and examined in Bruker S-3400N at a magnification of 10000 × and a voltage of 10Kv.

Results and Discussion

Species of *Aspergillus* are found to be widely distributed worldwide. They grow over a wide temperature range with relatively high humidity favorable for the fungi to thrive on leather.[34] The screening study reveals the dominant occurrence of genus *Aspergillus* with 58% of infested leathers screened.

Phenotypic characterization

Identification for all the 35 fungal isolates was carried out on the basis of their phenotypic morphology by visual observation with the help of taxonomic key as reported previously[18, 31-32] as well as by microscopic characteristics as studied by the classical method widely used for identification.[18] The macro morphological features for the selected fungal species are depicted in Table II.

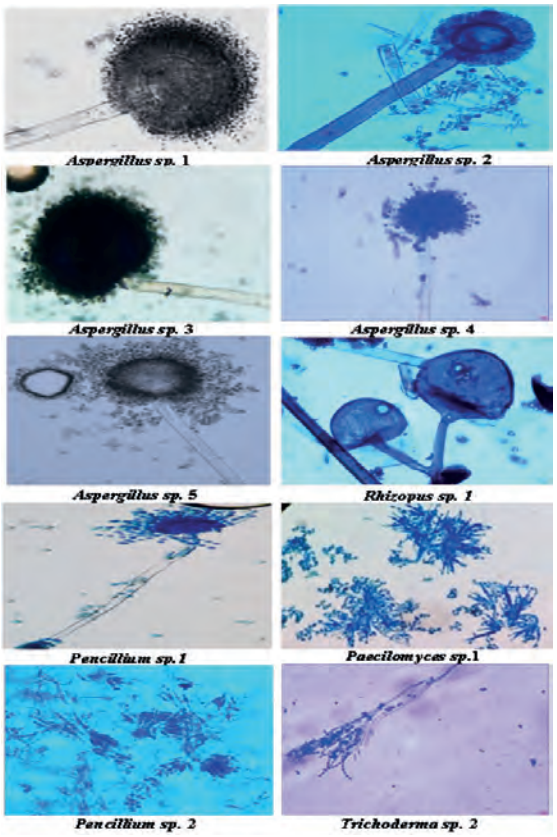

Figure 3. Lacto Phenol Cotton Blue stained fungal images observed at 40X magnification

Table II

Macro-Morphological features of the fungal isolates from tanneries

	MACRO-MORPHOLOGICAL FEATURES							
Species	**Surface/ Colony Color**	**Margin**	**Colony Reverse Color**	**Elevation**	**Growth**	**Production of Exudates**	**Soluble Pigments**	**Optimum Temperature & pH**
Aspergillums sp.1	Black (or) slightly brown to black	Entire	Creamy white to yellow/ black	Umbonate, rough surface	Moderate to rapid	Nil	Yes/some lack	32-37°C pH 5.5-7.0
Aspergillums sp. 2	Dark black	Entire	White to dull yellow	Umbonate	Moderate to rapid	Nil	Not known	32-35°C pH 5.0-6.5
Aspergillums sp. 3	White to yellow	Half or more	Pale yellow	Umbonate	Moderate	Yes	Yes	32-37°C pH 6.0-7.0
Aspergillums sp. 4	Yellow to green	Entire	Yellow to orange	Raised	Rapid and floccose	Yes/some lack	Yes	25-30°C pH 5.5-7.0
Aspergillums sp. 5	Dark black	Entire	White to pale yellow	Umbonate	Moderate	Not known	Yes	32-37°C pH 5.0-7.0
Penicillium sp.1	Olive velvety green	Half to entire vesicle	Pale to dark brown	Umbonate, velvety surface	Slow to moderate	Presence	Yes	25-35°C pH 4.5-6.5
Rhizopus sp.	Grey to black	Entire	Greyish black	Raised, even	Rapid	Yes	Yes	27-30°C pH 4.0-9.0
Paceliomyces sp.	Yellowish brown to tan color	Entire	Yellow to tan	Flat, powdery surface	Slow to moderate	Yes	Yes	30-35°C pH 4.5-6.5
Penicillium sp.2	Green with yellow border or fluffy white	Half or more	Pale yellow or dull white	Umbonate/ raised even	Rapid	Nil	Yes/some lack	32-37°C pH 4.5-5.6
Trichoderma sp.	Dark green with concentric rings	Curled	Deep yellow/ uncolored	Raised fluffy	Moderate to rapid	Not known	Yes/some lack	25-30°C pH 5.0-6.5

Table III

Micro-Morphological features of the fungal isolates from tanneries

Species	MICRO-MORPHOLOGICAL CHARACTERIZATION									
	***Aspergillums sp.* 1**	***Aspergillums sp.* 2**	***Aspergillums sp.* 3**	***Aspergillums sp.* 4**	***Aspergillums sp.* 5**	***Penicillium sp.* 1**	***Rhizopus sp.***	***Paceliomyces sp.***	***Penicillium sp.* 2**	***Trichoderma sp.***
Hyphae	Branched septate	Branched septate	Branched septate	Branched septate	Branched septate	Branched septate	Unbranched	Verticillated branched	Branched or unbranched	Unicellular with short hyphae
Conidiophore • Length • Diameter	 200-450 µm 11.5-15.0 µm	 — —	 500-2000 5.0-8.0 µm	 400-800 µm —	 950-1700 1.2-13.5 µm	 200-500 9-16	 210-300 µm 5-18 µm	 — 4.0-8.0 µm	 200-400 µm 2.6-4.0 µm	 — —
Vesicle	Globose	Globose to subglobose	Globose, radiate or columnar	Globose to subglobose	Globose or subglobose	Subglobose to ellipsoidal	Subglobose or oval	Subspherical to pyriform	Subglobose/ globus/ ellipsoid	Globose/ ovoidal shape
Conidia • Conidial heads • Diameter	 Blackish brown 3.5-6.0 µm	 Black 5.0-7.0 µm	 Rough 3.5-6.0 µm	 Smooth to rough —	 Rough 72-127 µm	 Rough walled 2.0-3.6	Black to pale brown 60-180 µm	 Smooth —	 Smooth wallled 2.1-3.2 µm	 — 2.6-3.0 µm
Ornamentation	Warty/ spiny	Echinulate	Slightly rough	Almost smooth	Rough, verrucose	Slightly Rough	Slightly rough to smooth	—	Smooth/ coarsely rough	Roughened or verrucose
Phialides	Biseriate coverentive vesicle	Uniseriate	Biseriated/ uniseriated	Mostly biseriated	Biseriated/ medulla vary	Biseriated phialides	Coenocytic	Verticillated branched/ cylindrical	Flask shaped phialides (ampulliform)	Philide sigmoid/ hooked
Fruiting bodies	Cleistothecium present	Cleistothecium present	Cleistothecium present	Cleistothecium present	Present	Cleistothecium present	Present	Present	Cleistothecium present	Present

All the isolates were stained with LPCB and observed at 40 × magnification under the microscope as shown in Figure 3 and the results of the microscopic characterization are detailed in Table III.

Depending on the colony color and texture, all the isolates were grouped into five different types which are presented in Table IV. However, a few of the strains from one sampling site exhibited resemblance to strains from other sites as well and hence were grouped together.

Table IV
Species-wise distribution of fungal isolates from tanneries

Groups	Unique Identification of the Isolates	Label for the Groups
Group 1	S-1, S-25, S-6, S-8, S-15, S-21, S-7, S-4	*Aspergillums sp.* 1
	S-5	*Aspergillums sp.* 2
	S-16, S-34	*Aspergillums sp.* 3
	S-10, S-24	*Aspergillums sp.* 4
	S-12	*Aspergillums sp.* 5
	S-20, S-29	*Aspergillums sp.* 6
	S-26, S-35	*Aspergillums sp.* 7
Group 2	S-17, S-2, S-30, S-14	*Paceliomyces sp.*
Group 3	S-11	*Penicillium sp.* 1
	S-23, S-27, S-31, S-13	*Penicillium sp.* 2
	S-33, S-19, S-28	*Penicillium sp.* 3
Group 4	S-3, S-18, S-32	*Rhizopus sp.*
Group 5	S-22, S-9	*Trichoderma sp.*

For example, group 1 represents *Aspergillus* species, which was further divided into seven sub types as identified by macro and microscopic examination. In the first sub type, the isolates, S-1, S-4, S-6, S-7, S-8, S-15, S-21 and S-25 were grouped under the umbrella of *Aspergillus* sp. 1 as the colonies exhibited black-brown dense colony with age and white-creamy colour at the circumference, revealing similarity amongst each other. The other sub types were grouped as follows. S-5 the lone member in the second sub type, the colony grew rapidly with black color spores and the reverse colony appeared to be wrinkled and yellow in color with age. The isolate S-12 had slow growth with white black colony with age and the mycelium appeared to be raised and rough. Though the S-12 colony morphologically resembled sub type 1 and 2, it did not have distinct conidiophore as viewed microscopically and hence was placed under third sub type. S-16 and S-34 isolates grew rapidly forming dense and homogenous colonies on the plate with pale green to brownish-yellow in color with age. S-10 and S-24 colonies appeared to be olive green in color when young and showed pale yellow in some areas with age. S-20 and S-29 colonies grew moderately, appeared initially white and exhibited velvety green-yellow color with age. S-26 and S-35 colonies grew moderately appearing green in color and reverse plate showing orange to brown with age.

The colonies of S-17, S-2, S-30 and S-14 appeared velvety powdery brown in color when young and the mycelium was flat, smooth and appeared furred with dark brown with age. All of them were grouped under *Paceliomyces* sp. (group 2). In group 3, *Penicillium* sp. isolates were again split into 3 sub-groups based on the colony morphology. S-11, the lone member in the first sub-group exhibited slow-moderate growth and the mycelium appeared white while young and showed velvety olive green with age. The second sub-group, S-13, S-23, S-27 and S-31 colonies appeared velvety to powdery with blue-green/

Table V

Fungal isolates obtained and their GenBank accession numbers

Fungal Strains	Accession Number of the Isolates (In this Study)	Closest Strain from Database and its Accession Number	% Similarity
A. niger (S-1)	MK372919	*A. niger* NJA-1 (KJ365316.1)	100%
A. niger (S-8)	MK372920	*A. niger* SF-6354 (KT185662.1)	99%
A. niger (S-6)	MK372922	*A. niger* strain F103 (MH299977.1)	99%
A. niger (S-4)	MK372923	*A. niger* strain F103 (MH299977.1)	99%
A. carbonarious (S-5)	MK372921	*A. carbonarious* strain CBS 127.49 (MH868003)	99%
Rhizopus oryzae (S-3)	MK372924	*R. oryzae* strain CBS 395.34 (MH867089)	99%
Paceliomyces variotii (S-14)	MK372925	*Paecilomyces* sp. WE3-F (KM874779.1)	99%
A. oryzae (S-9)	MK372926	*A. oryzae* strain SEMCC-3.248 (HM064501)	99%
A. nomius (S-10)	MK372927	*A. nomius* strain H5 (JF416646)	99%
A. versicolor (S-11)	KX814964	*A. versicolor* strain KBP MGU E61 (AJ9377	99%

grey-green often with white edge having red to brown exudates on surface with age. Third sub-group, S-19, S-32 and S-33 colonies grew moderately with white fluffy mycelium when young and white cottony in texture with age. The isolates of S-3, S-18 and S-32 in group 4 were labelled as *Rhizopus* sp. as the colonies showed white mycelium when young and grew rapidly with white-black furry appearance with age. The isolates of S-9 and S-22 were labelled as *Trichoderma* sp. (group 5) and the colonies in both cases appeared to have concentric ring like growth with yellowish- green with age.

From the macroscopic and microscopic observations, *Aspergillus* sp. was found to be the most dominant genus with 58% of the leather samples infested with this family of fungi followed by *Penicillium* sp. (18%), *Paceliomyces* sp. (11%), *Rhizopus* sp. (10%) and *Trichoderma* sp. (5%). Many species have been isolated and identified during various stages in leather processing by Sharma and Sharma.[33] The *Aspergillus* species was reported to be the most dominant fungi found to grow on leather, and they are known to produce asexual and sexual spores that disperse in air to long distances.[34] Among *Aspergillus*, *A. niger* was found to be the most dominant species, and this finding is in agreement with what has been reported earlier by Kanagy *et al.*[35] and Ozdilli *et al.*[36]

Genotypic characterization

Depending on the origin of the ribosomal operon (18s rRNA), gene homology (95-100%) of the species were identified. Ten isolates including some of the rare and commonly encountered fungal species were selected for identification. The obtained consensus sequence was compared for similarity with BLAST alignment search tool of NCBI Genbank dataset and evolutionary history was inferred using neighbor-joining method. The evolutionary analysis was carried out in MEGA 7.0 and distance in terms of number of base substitutions per site was computed using the maximum composite likelihood method.[25] All ten isolated strains and their sequences were deposited to Genbank database at NCBI. Their accession numbers and their closest strains from the database are given in Table V.

Phylogenetic analysis of all the ten strains was performed, among which four strains of *Aspergillums* sp. S-1, S-4, S-6 and S-8 belonged to *Aspergillums niger*, and they were grouped under a single tree as shown in Figure 4. Strain S-1 showed 100% identity to *A. niger* strain NJA-1 (KJ365316.1) from the dataset. Strains, S-4 and S-6 were rooted to *A. niger* strain F103 (MH299977.1) with 99% identity and the closest relative is *A. niger* isolate SB2 (MH041197.2). Strain S-8 was rooted to *A. niger* strain SF-6354 (KT185662.1) with 99% identity.

The other isolated strains of *Aspergillus* sp. S-5, S-10 and S-9, *Rhizpous* sp. S-3 and *Paecilomyces sp.* S-14 have been divided into 4 clusters as shown in Figure 5 for ease of presentation. Cluster I consisted of S-9 belonging to *A. oryzae* and S-10 to *A. nomius*, along with closest reference strains of *A. oryzae* SEMCC-3.248 (HM064501), *A. nomius* H5 (JF416646) and *A. nomius* (AB008404) respectively. Cluster II consists of S-14 strain belonging to *Paecilomyces variotii* along with its closest neighbour *Paecilomyces* sp. WE3-F (KM874779.1) and *Parcilomyces variotii* JF416647.1 with 99% identity.

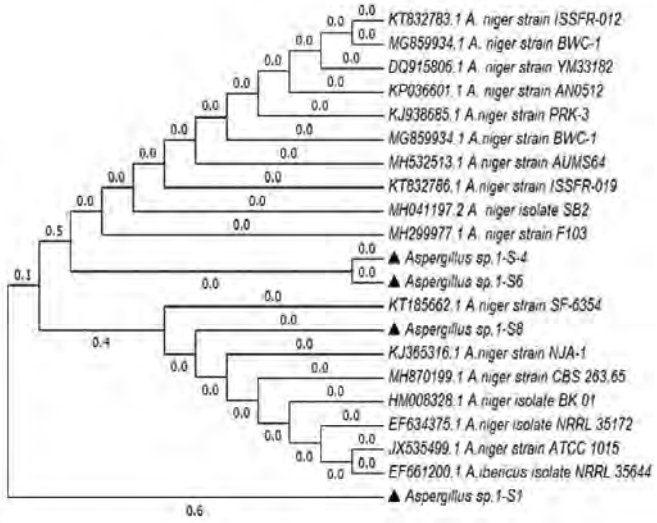

Figure 4. Phylogenetic tree constructed with 21 strains of *A. niger* nucleotide sequences, by Neighbour joining method (NJ) method for *Aspergillus sp.* (S-1, S-4, S-6, S-8). The values represented on the branch specify branch length with optimal tree length of 1.55 having 528 positions in final dataset

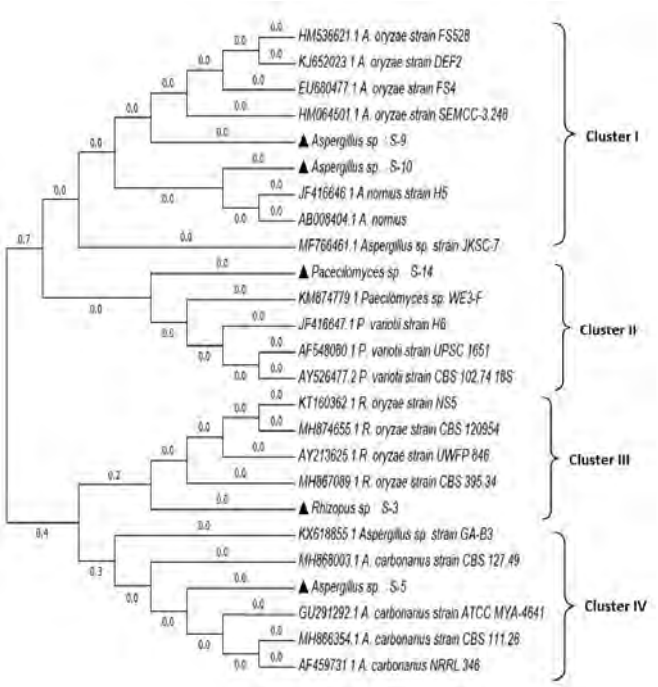

Figure 5. Phylogenetic tree constructed by Neighbor joining method (NJ) for *Aspergillus sp.* S-5, *Aspergillus sp.* S-10, *Aspergillus* sp. S-9, *Rhizopus* sp. S-3 and *Paecilomyces* sp. S-14, involving 25 nucleotides with 468 positions in the final data set. The values presented on the branch specify the length with optimum tree with the sum of branch length of 9.60

Cluster III consist S-3 strain rooted to *R. oryzae* CBS 395.34 (MH867089) with 99% similarity along with reference strains *R. oryzae* AY213625.1, MH874655.1 and KT160362.1. Cluster IV consists of S-5 strain rooted to *A. carbonarious* CBS 127.49 (MH868003) strain with 99% similarity, with other closest neighbours being strains of GU291292.1, MH866354.1 and AF459731.1 of *A. carbonarious.* Out of ten species, four strains (S-1, S-4, S-6, S-8) from *Aspergillus* sp. 1 group were identified as *Aspergillus niger.* S-5, S-9 and S-10 were identified as *Aspergillus carbonarious, Aspergillus oryzae* and *Aspergillus nomius* respectively. S-3 belonged to *Rhizopus oryzae* and S-14 to *Paecilomyces variotii.* S-11 which was grouped under *Penicillium* sp. based on phenotypic characterization earlier was found to be *Aspergillus versicolor* on gene sequencing. The results of the evaluation studies on susceptibility of this strain to commonly used leather biocides have been reported elsewhere.[37]

***In vitro* susceptibility of the fungal strains against selected biocides**

Susceptibility test for the selected ten isolates was carried out against four structurally different biocides namely, 2-(thiocyanomethylthio) benzothiazole (TCMTB), 2-Mercaptobenzothioazole(2-MBT), 3-iodo-2-propynyl-N-butylcarbamate (IPBC) and 2,2-dibromo-3-nitrilopropionamide (DBNP) (Figure 6) commonly employed in leather industry.

The biocides were used in the range of 0.5-1000 μg ml^{-1} to test their efficacy to control the growth of fungi. The MICs for the selected isolates against biocides are presented in Table VI. All the experiments were conducted in triplicate and the two concordant inhibition concentrations were taken as MIC.

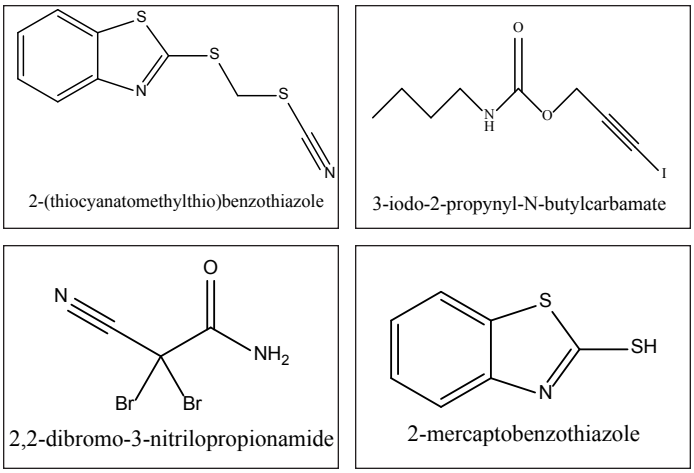

Figure 6. Chemical structures of leather biocides used in this study

Table VI

Evaluation of selected biocides against the isolated fungal strains

	Biocides (μg/ml) concentration			
Fungal Isolates	**TCMTB**	**2-MBT**	**IPBC**	**DBNP**
A. niger ATCC 6275 (Wild type)	1.95	125	3.90	125
A. niger (S-1)	3.90	125	1.95	250
A. niger (S-8)	0.97	250	1.95	250
A. niger (S-6)	7.81	250	3.90	125
A. niger(S-4)	1.95	31.25	3.90	125
A. carbonarious (S-5)	0.97	7.81	7.81	15.62
Rhizopus oryzae (S-3)	0.97	7.81	3.90	3.90
Paceliomyces variotii (S-14)	31.25	125	125	250
A. oryzae (S-9)	31.25	62.5	125	15.62
A. nomius (S-10)	1.95	125	62.5	125
A. versicolor (S-11)	31.25	250	250	62.5

From the results, TCMTB was found to be the most effective biocide in inhibiting the growth of fungi with least concentration when compared with other biocides. The MIC concentration of TCMTB was highest for strains S-9, S-11 and S-14 (31.25μg ml^{-1}) followed by S-6 (7.81μg ml^{-1}), S-1 (3.90 μg ml^{-1}), S-4, S-10 and wild type (1.95 μg ml^{-1}). The least inhibition concentration was observed for the strains S-8, S-5 and S-3 (0.97 μg ml^{-1}).

Among the four biocides, DBNP was required in high concentration for inhibition of fungal growth. The following decreasing trend of MIC for different strains was observed: S-11 (625 μg ml^{-1}) > S-1, S-8 and S-14 (250 μg ml^{-1}) > S-4, S-6 and wild type (125 μg ml^{-1}), S-5 and S-9 (15.62 μg ml^{-1}) > S-3 (3.906 μg ml^{-1}). With 2-MBT, highest MIC was observed for strains S-6, S-8 and S-11 (250 μg ml^{-1}) and

the least for S-3 and S-5 (7.8125µg ml^{-1}). In the case of IPBC, strain S-11 required highest concentration of 250µg ml^{-1} whereas S-1and S-8 required lowest concentration of 1.953µg ml^{-1}.

After the susceptibility test, the vials with TCMTB concentrations, MIC and above MIC were further subjected to identify the minimum fungicidal concentration (MFC). The MFC that required to kill the cells was the highest for S6 *(*152 µg ml^{-1}) when compared with that of the other isolates and wild type strain (31.25 µg ml^{-1}). From the results, it is concluded that S-6 is the most tolerant or resistant among all five *Aspergillus niger* strains and S-4 resembles very closely to wild type strain. TCMTB was the most effective, as the concentration required to inhibit the growth of fungi was the least compared to other biocides. The effectiveness of TCMTB on tanned leathers and its wide usage in leather industry has been reported earlier.[5, 38] From the susceptibility test, strain S-6 required four-fold increase in concentration (7.813µg ml^{-1}) of TCMTB compared to the wild strain (1.95µg ml^{-1}) and hence concluded to be the most biocide tolerant strain.

Based on the study, the *Aspergillus niger* isolates, S-6, S-1, S-4 and S-8 which required high concentration of TCMTB for inhibition were selected for further study.

SEM Analysis

Guarro *et al*.[39] have used SEM analysis to identify fungi of the same genus to their species level. Silva *et al*.[40] used the technique to identify species of *Aspergillus* genus within *Aspergillus* section *Nigri* complex. But in this study, SEM studies were carried out to understand the differences, if any, in the morphology of conidia and conidiophore due to evolutionary changes in the strains due to probable resistant development. SEM analysis revealed that all the isolates and the wild type *A. niger* have similar conidiophores, which appear to be smooth, long and narrow as shown in Figure 7. *A. niger* species S-1, S-4 and S-8 and corresponding wild type had similar morphology with round vesicle and radiated head when compared with S-6 strain, which showed conglomerated conidia (Figure 7d) with higher mycelial aggregation and sporulation.

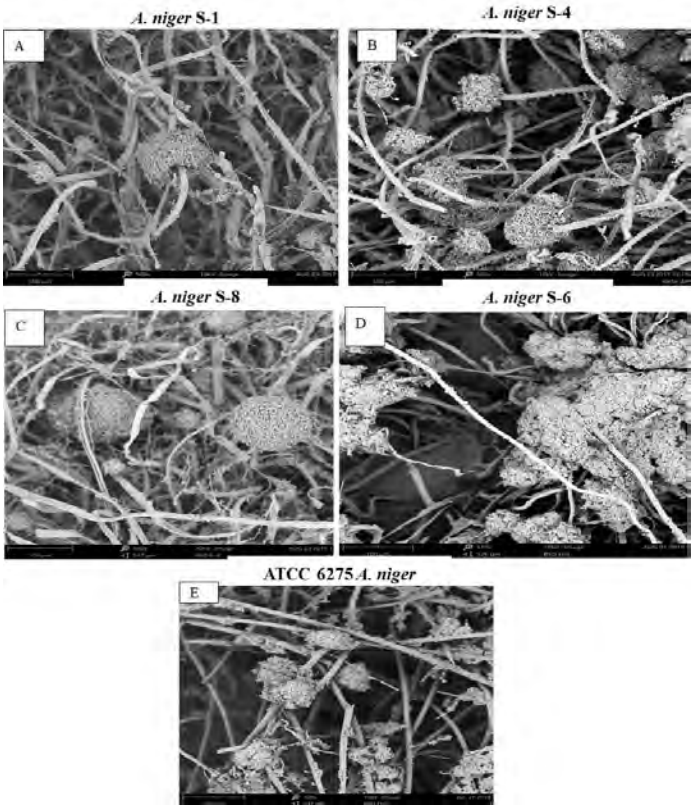

Figure 7. Scanning Electron Microscopic images of *A. niger* at 500 X magnification. (A-D) Isolated strains *A. niger* and (E) Wild type *A. niger* (ATCC 6275).

The isolates S-1, S-4, S-8 and wild type appeared to have round and radiated vesicle with biseriated phialids which was not distinctly visible in the case of S-6 strain. These observations might give further support that S-6 may have developed some sort of tolerance/ resistance to the biocide.

Evaluation of growth of *A. niger* strains on biocide treated wet-blue Leathers

Study was conducted to identify the *A. niger* strain which could resist biocide action the most (Figure 8). The wild type *A. niger* showed slight growth on 32nd dwith 0.2% TCMTB as shown in Figure 8D.

Among the test strains, *A. niger* S-6 was found to be the most dominantly growing which showed a slight growth on the grain

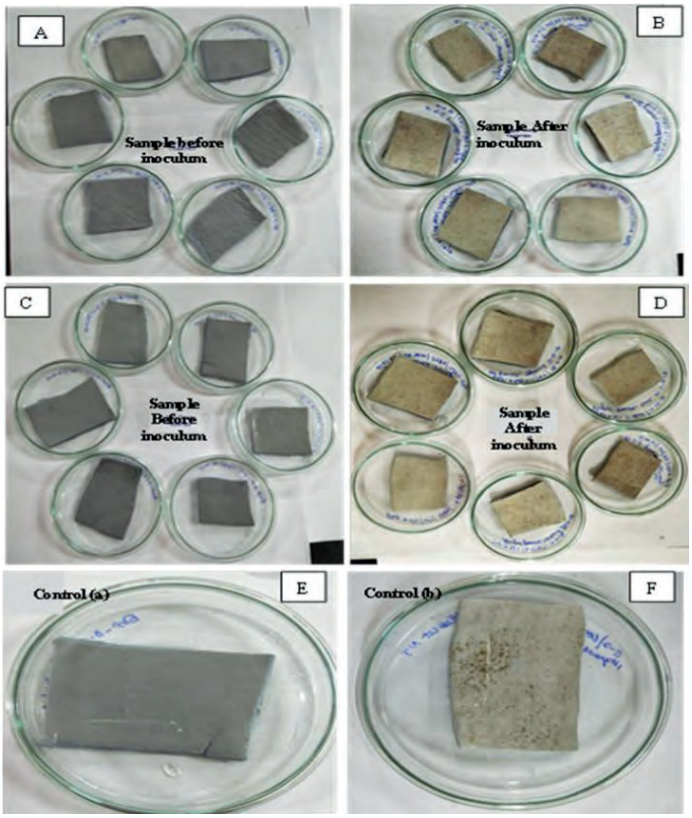

Figure 8. Leathers treated with TCMTB and evaluated for the growth of fungal resistance. A & C are biocide treated leathers before inoculum, B & D are treated leathers inoculated with *A. niger* S-6 and *A. niger* wild type respectively. Piece treated with 0.2% TCMTB served as control (a) without fungal inoculum and (F) is the growth control (b) without any biocide treatment

surface on 8thd of inoculum with 0.05% TCMTB, 11th d with 0.1%, 18th d with 0.2% as shown in Figure 8B. With S-6, the growth was the highest with the grain area being completely covered and the flesh side showing sparse growth. Even with high concentration of biocide (0.75%), growth was seen in the cross section of the leather on 45th d. S-1, S-4 and S-8 isolates showed slight growth on 11th d for 0.05% TCMTB. S-4 showed moderate growth on 40th d for 0.2% TCMTB and no growth with 0.75%, whereas, S-1 and S-8 showed growth on 25th and 30th d respectively for 0.2% TCMTB. As per the published literature, about 0.2% offering of TCMTB could give adequate protection for the wet blue leathers stored for 3-4 months.[5] But this accelerated susceptibility study indicates that all the isolates studied in this investigation have gained some sort of tolerance against TCMTB and among them S-6 seems to be the most resistant as it was found to grow even at very high concentration of 0.75%, which is not usually employed in leather processing. Therefore, *in vivo* growth studies on chrome tanned biocide treated leathers provided further support to the finding that the strain S-6 was the most resistant with persistent growth.

Conclusion

From the present screening study, it is revealed that *Aspergillus* sp. was the most dominant fungal species found to grow despite biocide treatment with an occurrence on 58% of the leather samples screened. Among *Aspergillus genus, Aspergillus niger* was found to be the most common and dominant species growing on leathers. From the results of *in vitro* and *in vivo* studies carried out with wild type *A. niger* as control, the isolate *A. niger* (S-6) was found to be the most biocide tolerant. It is proposed to carry out detailed studies with this particular strain to understand the resistant mechanism.

Acknowledgement

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Influence of Benzenecarboperoxoic Acid on Chamois Leather Process

by

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Abstract

Stabilization of collagen against heat and enzyme is a key objective in the tanning process. In oil tanning, fatty acid present in the oil is oxidised mainly into aldehydes which interects with Ɛ amino groups of collagen to form stable covalent cross links. Conventionally, oil tanning consumes time from two to three weeks which primarily depends on the type of oil and oxidation method for completion of tanning. In the present research, the duration of oil oxidation is reduced using benzenecarboperoxoic acid (PBA). It has been observed that PBA significantly reduces oil tanning duration from two weeks to 4 days. Moreover, the water absorption capacity of experimental leather has also increased by approximately 48% (1% PBA) compared to control leather. Physical strength properties such as tensile and percentage elongation values have also found to meet the standard norms. In addition to this organoleptic properties are also on par with control leather. The present study focus on the accelaration of chamois process for making leather, using PBA as an oxidising agent.

Introduction

Hides/skins are converted to leather finds valuable application in numerous fields.[1] The conversion of skin/hide into leather proceeds with several chemical changes in the collagen structure. The core of the leather making relies on the permanent preservation process called tanning, by treating skin/hides with suitable chemicals depending on the type of leather to be produced.[2] Some important properties of leathers such as strength, elasticity, fluffiness, waterproofing can be imparted by the use of oil and waxes during post tanning and finishing process respectively.[3] Commercially chrome and vegetable tanning are widely used because of their multifunctionality. Tanning system such as aldehyde base system is used for selective leather products. Oil tanning is a traditional method for manufacture of leathers with high water absorption capacity which are commercially known as chamois leather. The properties of chamois leather make its exclusive for applications such as cleaning, drying, filtration and apparel making.[4] Chamois leather finds applications in various other fields such as filtration of high-grade gasoline, cleaning of optical instruments, manufacturing of gloves and garments, for lining trusses and prosthetic devices, sweat and water/oil absorbent applications.[5]

Commercially, chamois leather manufacture is a time-consuming process which primarily depends on the atmospheric oxygen to oxidise the oil during tanning to convert fatty acid into aldehydes which intern reacts with leather making protein collagen. During oil tanning, oil is applied on the skin and hooked to atmospheric condition to complete the tanning process. Several attempts have been tried to reduce the oxidation duration, thereby increasing the production rate.[6,7] The process of making chamois is time consuming, the long duration of chamois process can lead to the adverse effect on production of chamois leather.[8-10] Hydrogen peroxide (H_2O_2), sodium per carbonate ($2Na_2CO3.{}_3H_2O_2$) and ozone (O_3) are commonly used for oxidising oil. However, handling of these materials needs care due to their strong oxidizing ability and corrosive nature.[11-14] Hence there is a need for alternative oxidizing agents which needs minimal care in handling and yields effective oxidation. Therefore, the current investigation focused on developing new chamois process using PBA as an oxidising agent.

Materials and Methods

Materials

Indian sheep skins were procured from the local slaughterhouse, Chennai, India. Glutaraldehyde, sodium carbonate, fish oil and all other leather chemicals were of commercial grade.

The preparation of Benzenecarboperoxoic acid or peroxybenzoic acid (PBA)

A solution of 8.0 g. (0.102 mole) of sodium peroxide in 135 mL cold solution of water not exceeding 20°C. The solution is filtered using filter paper to remove the yellow suspended solids. The filtrate is placed in a 1000 mL beaker under stirring, then 175 mL of ethanol and a solution of 0.5 g of hydrated magnesium (in 15 mL of water) were added. The solution was brought to room temperature (37°C) and 11.6 mL (0.100 mole) of benzoyl chloride was added drop wise under constant stirring. The mixture was filtered to remove benzenecarboperoxoic acid. The filtrate was acidified with 20% of sulfuric acid and extracted with carbon tetrachloride. Six

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Table I

Detail description of process for making chamois leather

Process	Chemical	Percentage (%)	Time (min)	Remarks
Washing	Water	100	10	Wash and drain
Deliming	Water	100		
	Ammonium chloride	2	40	
				Check deliming using phenolphthalein
	Alkaline bate	0.5	30	Drain
Washing	Water	200	10	Wash and drain
Partial pickling	Water	80		
	Salt	8	30	
	Formic Acid	0.5	30	In 1:10 dilution with water
	Sulphuric Acid	0.2		In three feeds with 1:10 dilution with water, adjust pH to 4
	Glutaraldehyde	1	60	Drain, pile for overnight
Next day				
	Fish oil	25		
	Benzenecarboperoxoic acid (experiment)	X		
	Sodium carbonate	0.5		Mix using stirrer, make paste. add to drum along with skin
X= 0.25, 0.5, 0.75, 1				

extractions using about 75 mL portions resulted into 0.075 mole of benzenecarboperoxoic acid.[15]

Experimental design of chamois making using fish oil

Limed pelts were delimed and pickled to a pH of 4. The fibers were pre-stabilised using glutaraldehyde, as the chamois process was carried out by exposing skins to air for two to three weeks based on colour change (oxidation). These pre-stabilised skins resist petrifaction and helps maintain the structural integrity of the skin matrix. The detail description of oil tanning has been mentioned in Table I. Chamois leathers were made by adding fish oil mixed with small quantities of sodium carbonate (control) and various percentages of oxidizing agent (experimental) along with skin in a rotatory drum for 90 min to achieve thorough distribution of oil to the skin, the leathers were hooked for drying.

After a period of 96 h, skins turn to golden yellow colour indicating the completion of oil tanning (experimental). For control, the air oxidation process continued with regular monitoring for 14-15 days until leathers turn to golden yellow. Then, the leathers were washed with water (100%), soda ash (1%) and wetting agent (1%) for the complete removal of unfixed oil. After drying, leathers were subjected to staking and milling.

Analysis of chamois leathers

Hydrothermal stability is an important parameter to know the leathers resistance towards heat. Shrinkage temperature of chamois was analysed according to standard procedure.[16] Phenom Pro desktop scanning electron microscope (SEM) was used to analyse the morphology of chamois leathers, the instrument was equipped with light and electron optical modes operating at 5 kV acceleration voltage. PMI capillary flow porometer was used to measure the air permeability. The pressure was varying from 0-20 psi. Physical strength parameters of leather were studied after sampling; tensile strength was analysed according to the standard procedure.[17,18] Values reported were average of four samples. Measurement of water absorption was carried out as per the standard procedure.[19] Organoleptic Properties were assessed for softness, colour and odour by subjective evaluation and the rating was awarded on a scale of 0-10 points and higher points indicate better properties. The evaluation was carried out by two experienced leather technologists.

Results and Discussion

Plausible mechanism of interaction of PBA with oil and followed by collagen during the chamois process

Fish oil mainly consist of unsaturated double bonds which are susceptible for auto oxidation during the chamois process. When exposed to air, molecular oxygen reacts with double bonds of fatty acid present in the oil leads to the formation of aldehydes, ketones and other derivatives.[20] An oxidising agent may enhance the oxidation process of fatty acid present in the oil. When benzenecarboperoxoic acid is used to oxidize oil, the oxygen from benzenecarboperoxoic acid interact with the double bond present in

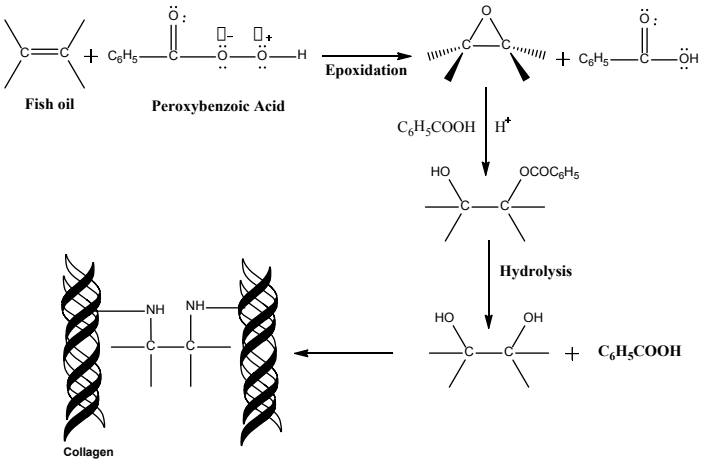

Figure 1. Plausible mechanism of oil tanning

Table II

Shrinkage temperature measurement of chamois leather

S No	Sample	Shrinkage Temperature (°C)
1	Control	78±1
2	PBA (0.25%)	78±1
3	PBA (0.50%)	79±1
4	PBA (0.75%)	79±1
5	PBA (1.00%)	80±1

Table III

Physical testing data of chamois leathers

S No	Sample	Tensile strength (N/mm2)	Percentage elongation (%)	Water Absorption (%)
1	Control	14±0.70	62±3.10	463±23.5
2	PBA (0.25%)	16±0.80	58±2.80	396±19.8
3	PBA (0.50%)	17±0.85	60±3.00	448±22.4
4	PBA (0.75%)	25±1.25	61±3.05	560±28.0
5	PBA (1.00%)	27±1.35	64±3.20	688±34.4

fatty acid though the formation of three-membered ring.[21] Further three membered oxirane ring by subsequent side reactions generate diols which can interact with Ɛ amino group of collagen matrix. The plausible mechanism of interaction of reactive species is shown in the Figure 1

Analysis of chamois leather

Hydrothermal Stability or Shrinkage temperature of chamois leathers were analysed to understand the irreversible deformation of leather under hydrothermal heat. From Table II, shrinkage temperatures of experimental leathers were in the range of 78-80 ±1°C, which is comparable to conventional oil tanning of 78 ±1°C. The reported values are measured for average of 4 samples.

Physical strength properties were analysed to understand the chamois matrix strength to withstand load. Leathers were analysed for tensile strength property measurement. From Table III, it can be seen that the tensile strength increased with increase in percentage of oxidizing agent. Another important functional property of chamois leathers is water absorption; from the table it can be observed that the percentage water absorption of chamois leathers increased by 48% (1% PBA) compared with control leathers made without use of oxidizing agent

Cross sectional morphological changes were analysed using SEM. Fiber alignment of control leathers have been found to be compact compared to experimental leathers. From Figure 2, it can be seen that the fiber alignment is more opened compared to control leathers.

The wider the aligned fibers the higher degree of porosity, which is a desirable quality of good chamois leathers.

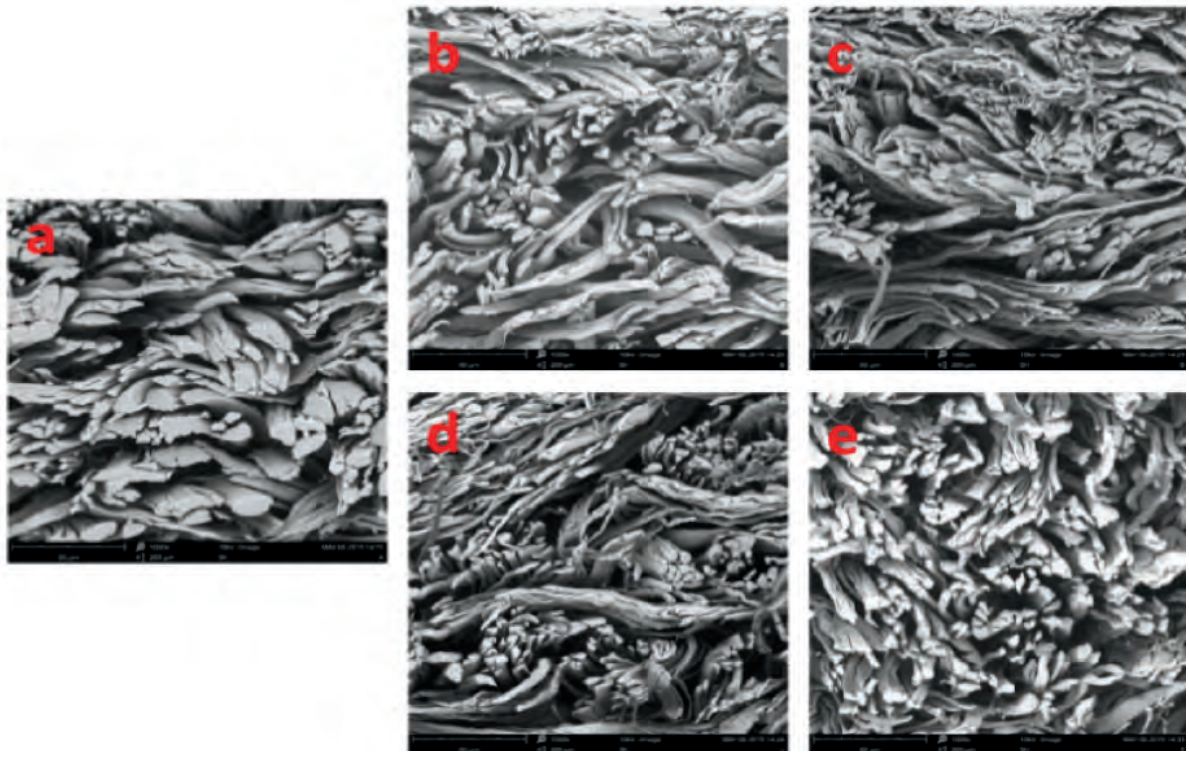

Figure 2. SEM images of chamois leathers control (a), PBA (0.25-b, 0.5-c, 0.75-d, 1%-e)

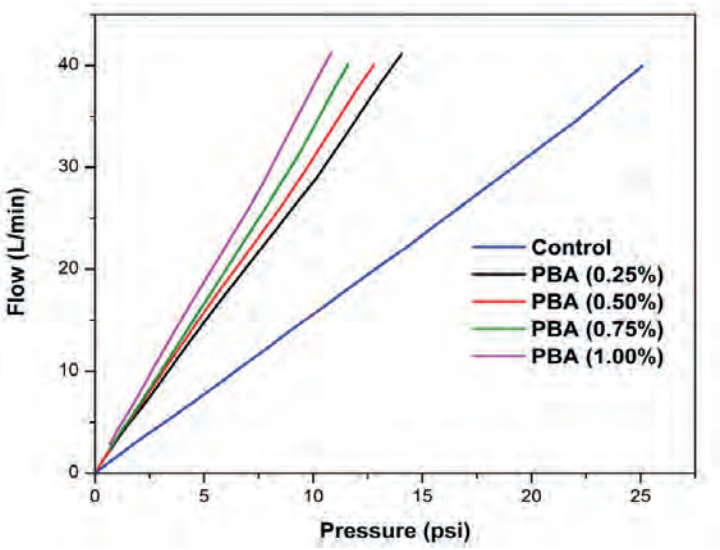

Figure 3. Porometry analysis of chamois leathers

Porosity of chamois leathers were analyzed to understand the pore structure of the matrix. From Figure 3, it can be observed that the porosity of the chamois leathers increased with the use of PBA with varying percentages.

With minimal pressure (1-12 psi) the air flow rate (liter per minute) of the experimental leathers increased drastically. It can also be safely concluded that the leathers made using the PBA exhibit good porosity comparable to control leathers, this can further have verified using SEM images for visual conformation, the alignment of fibers can be observed to be more open for experimental leathers.

From Figure 4, the organoleptic properties of chamois leathers were assessed for softness, color and odor. It can be seen that the softness of chamois leathers improved when higher concentrations of oxidizing agent were used for chamois making. The color of the experimental chamois leathers did not show any effect with variation of the PBA percentage. As the chamois leathers are made using fish oil, odor is one of the main concerns on the final leathers, though the unreacted fish oil from the process is removed by alkali washing, small quantities remain and contribute to odor.

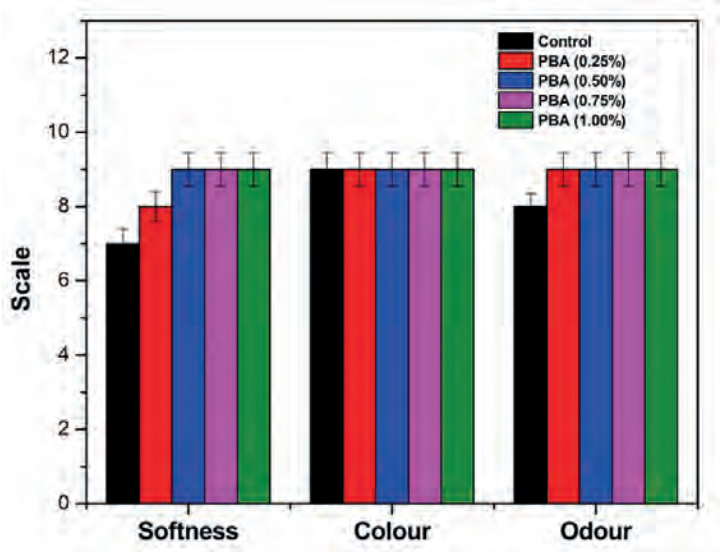

Figure 4. Bulk properties of control and experimental chamois leathers

Conclusions

The present study provides an insight on the application of benzenecarboperoxoic acid to reduce the duration of oxidation of oil for chamois manufacture. The use of benzenecarboperoxoic acid as an accelerant to oxidize fish oil in chamois making process reduces the process duration from 15 to 4 days. Water absorption capacity has also been increased by approximately 48 % (1% PBA) as compared to conventional chamois leathers. Further, shrinkage temperature of the chamois leathers was comparable with control leathers along with other organoleptic properties such as softness, color and odor. Hence, it can be concluded that the use of benzenecarboperoxoic acid in chamois making not only reduces the time but also have positive benefits on the physical properties of chamois leathers.

Acknowledgements

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Limiting Microbial Activity as an Alternative Approach of Bovine Hide Preservation Part II: Impact of Developed Formulations on Leather Quality and the Environment

by
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Abstract

Wet salting of bovine hide commonly utilizes 95% saturated salt solution or 40-50% salt (w/w) on raw hide weight for preservation. The salt used for the hide preservation ends up being in wastewater and generates enormous environmental pollution. To minimize the environmental pollution problem associated with the traditional method of hide preservation, alternative formulations containing antimicrobial agents and less amount of common salt (35% saturated brine) have been developed. The alternative formulations were found to be more effective in deterring microbial growth than the traditional formulation as demonstrated by the total aerobic bacterial count of the preserved hide soaking liquor. The effect of the newly developed formulations on leather quality was assessed by analyzing the mechanical properties, scanning electron microscopic images, grain pattern and organoleptic properties of the finished leather. The quality analysis of the crust leather revealed that, the leather panels produced from the traditionally and alternatively preserved hides were comparable. The environmental impact of the newly developed formulations was also evaluated by monitoring the leather processing effluents for the pollution indicators such as total solids (TS), total dissolved solids (TDS), chloride content, Chemical oxygen demand (COD) and Bio-Chemical oxygen demand (BOD). Overall, the environmental impact of the newly developed hide preserving formulation was less severe than the traditionally used formulation. Since the newly developed formulations did not affect the quality of the leather produced and their impact on the environments is minimum, they could be considered as viable options for combatting pollution problems associated with the traditional salt curing method.

Introduction

Bovine hides and skins are non-edible byproducts of the meat industry and they are the main raw materials for leather production. Preservation of raw hides is the first step in the leather production process and it must start right after the raw hide is collected from the slaughterhouse, otherwise the hide will start losing its integrity because of putrefaction. The major component of fresh hide is moisture (60-70%) and the proteins (collagen, elastin, keratin) constitute about 30% of the hide. The integrity of raw hide is an important factor in producing a good quality leather. A variety of microorganisms are found on animal hide and these microorganisms come from different sources such as air, water, soil, manure and others.[1,2] The natural defense system protects skin of living animal from being attacked by bacteria and other microorganisms but the flayed skins become a source of bacterial growth within 5 to 6 h of removal because of the favorable moisture conditions.[3] The normal microflora growing in a hide produces proteolytic and collagenolytic enzymes that damage structural proteins resulting in deterioration of hide. Since the quality of the produced leather relies on the amount of important proteins in raw hide, it is vital to protect proteins from decomposition during hide preservation process.[4]

The main objective of hide preservation is to prevent putrefaction and this is achieved by either killing the microorganism (using bactericidal agent) or by inhibiting the growth of the microorganisms (using bacteriostatic agents) on hides. The bactericidal agents used to kill the microorganisms are usually expensive and not safe to humans or living species. Bacteriostatic agents are dehydrating agents commonly used in bulk like sodium chloride which is responsible

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for generating salt pollution problems. Pollution problem is assessed by measuring total dissolved solid (TDS) and chloride content in the salt bearing effluents that come from leather processing facility because of the soaking operation.[5]

Traditionally, hide curing is accomplished by soaking the raw hide in 95% saturated salt solution. Close to three fourth of the salt used goes to the discharged stream during soaking, which contributes to more than 40% of total dissolved solids in the tannery wastewater creating huge amount of salt pollution in the environment.[6,7] When the tannery wastewater is dumped to the open field, the high salinity prevents seed germination and the growth of seedlings and other floras.[8] Furthermore, when soils are watered with saline waste, salt is likely to accumulate in the field due to low-soil permeability, inadequate drainage and rainfall. The accumulated salt has a harmful effect on crop production and seriously impacts the environmental.[9,10,11] The discharged salt also causes adverse effect on quality of water and growth of plants by influencing the solubility and release of heavy metals.[12,13,14] Therefore, it is very important to develop eco-friendly hide preservation method where no salt or less salt is utilized.

Many researchers have been actively involved in the development of alternative hide preservation methods with less environmental impact. The alternative methods that have been published could be categorized into the physical and chemical methods of curing hides.[15] The physical methods include cooling,[16] cooling in vacuum,[17] drying chamber[18] and gamma rays or electron beam based irradiation.[19,20] Even though these physical methods are environmentally friendly, most of them are expensive and they have not been adopted in a hide processing facility. For the chemical methods, the chemicals that have been explored for hide preservation include potassium chloride,[21] soda ash,[22] benzalkonium chloride,[23] antibiotics,[24] neem oil[25] and boric acid.[26] However, because of various reasons such as high cost, toxicity, inadequate curing efficiency, severe environmental impact and poor leather quality, most of the chemicals have not found commercial application.

As part of our effort to develop an alternative and environmentally friendly curing method, we previously reported[27] the effectiveness of 35% saturated brine solution in combination with three different biocides in preserving hides for more than a month. This method decreased salt usage by around 60% when compared to the commonly used 95% saturated salt solution. The effectiveness of the developed formulations on hide preservation were evaluated and reported by monitoring different parameters including water activity, microbial growth, texture, moisture content and microscopic analysis of the cured hide during the preservation period. The developed formulations were found to be effective in limiting bacterial growth, controlling yeast and mold growth during more than 30 days of storage.[27] This study is a continuation of the previously reported findings and here we report the effect of the developed formulations on produced leather quality and the impact of the formulations on the environment compared to the conventional hide preservation method.

Experimental

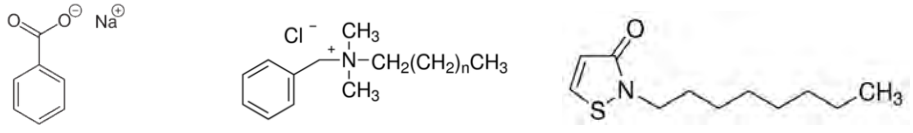
Materials:

Fresh bovine hides were obtained from the local meat packing company, JBS Packerland (Souderton, PA). Each hide was cut into smaller pieces of equal size (12 in x 12 in) that weighed approximately 420–700 g. All the compounds used to prepare the formulations are described in Table 1. They were purchased from vendors as mentioned in part 1 published article[27] and they were of laboratory grade at the time of use. Brine solutions were prepared by dissolving the required amount of salt in tap water at room temperature and the saturation level was confirmed with a salometer. The formulations were prepared as described in Table I by dissolving the chemicals in tap water at room temperature (~22°C) and ~12 h prior to conducting the experiments.

Table I

Composition of the developed hide preserving formulations

Formulations	Composition
F-A (control)	95% saturated brine soln. + 0.043% NaOCl (v/v)
F-B	35% saturated brine soln. + 0.4 % ŧBAC (v/v) + 0.25% ŧNa-B
F-C	20% saturated brine soln. + 1% LA + 0.25% Na-B
F-D	35% saturated brine soln. + 1% LA + 0.25% Na-B + 0.15% NaOH + 0.1% Tween 20
F-E	35% saturated brine soln. + 1% LA + 0.25% ŧOIT + 0.1% Tween 20
F-F	35% saturated brine soln. + 1% LA + 0.4 % BAC (v/v)



ŧNa-B: *Sodium benzoate* ŧBAC: *Benzalkonium chloride* ŧOIT: *2-n-octyl-4-isothiazolin-3-one*

Laboratory Scale Hide Preservation Protocol

The hide pieces were cured by soaking them in 150% float (volume of solution/weight of hide) separately in a 6-in-1 Dose drums which was set to tumble the hide at 6 rpm (Dose Maschinenbau GmbH, Lichtenau, Germany) for 18 h. The control was treated with saturated salt solution (95%) and 0.043% (v/v) bleach (NaOCl) (F-A, Table 1) which is the traditional hide preserving formulation. For the alternative formulations (F-B to F-F, Table 1), a 35% saturated salt solution with different antimicrobials was used in common except F-C, where 20% saturated brine was used. After allowing the treatment for 18 h, extra water was removed from the hide surface and then, they were folded and stored in a humidity chamber at 38-40°C. The hide pieces were periodically observed for possible physical changes such as smell and hair slip which would indicate hide deterioration. The effectiveness of the new hide preservation formulations was evaluated by determining different parameters as described in our previously published studies.[27]

Analysis of Soaking Liquor Generated in Leather Processing

After 35 days of preservation, the hide samples were soaked with 200% (v/w) float of water for 4 h. Then, the soaking liquor of the differently preserved hides was quantitatively measured and analyzed for various pollution load parameters including total solid (TS), total dissolved solid (TDS) and chloride (Cl^-) content according to accepted analytical procedures.[28,29,30,31] Aerobic bacterial colony count, Total carbon (TC) and organic carbon (TOC), biochemical oxygen demand (BOD) and chemical oxygen demand (COD) of soaking liquid were also measured to evaluate the overall pollution load resulting from the soaking operation, the first step of leather processing.[28]

Aerobic Bacterial Count of the Soaking Liquor

The soaking liquors from the preserved hide pieces were collected separately and placed in sterile vessels to determine residual aerobic bacterial concentrations. Sterile water was used to prepare serial of dilutions of the collected 1 ml soaking solution. The diluted soaking liquor sample was spread on Tryptic Soy Agar plate and incubated at 37°C for ~24 h. The bacterial colonies were enumerated on the agar and the bacterial population is reported in log CFU/ml. All soaking liquor samples were repeatedly checked three times.

Yeast and Molds Colony Count of Soaking Liquor

Previously published procedure[27] was followed to count yeast and molds colonies in the soaking liquors. Dichloran Rose Bengal chlortetracycline (DRBC) agar was used for this analysis, where DRBC is selective for yeast and mold growth and inhibits bacterial growth. Yeast and molds colonies were enumerated after 4 days of incubation at 22-25°C and expressed as log CFU/mL.

Measurement of TOC and COD in Soaking Liquor

TOC and COD of the spent liquor from the soaking operation were quantitatively determined according to the procedure described in our previous article.[28] The results are reported in terms of g/kg of cured hide.

BOD Analysis of Soaking Liquor

The biochemical oxygen demand (BOD) of soaking liquor represents the amount of oxygen utilized by the microorganisms (biochemical processes) to degrade the organic matters present in the sample. This is a parameter of measuring biodegradable materials in soaking solutions. The BOD measurement was conducted for 5 days according to the previously published procedure.[28]

Tanning Operation of Cured Hides

Following the soaking operation, the hide pieces cured with developed formulations were put in one hair removing drum and the control was put in a different drum. The dehairing process was carried out according to the USDA tanning protocol.[32,33,34] Then all the hide samples were put into a single drum for pickling, tanning, re-tanning, coloring, and fat liquoring steps. The cured hides were processed into crust upper shoe leather and stored in a controlled environment, where the temperature and humidity are maintained at 21°C and 50% (relative humidity) respectively, until the quality tests were performed as described below.

Determination of Leather Quality

The effect of the newly developed hide preserving formulations on leather quality was determined by measuring the mechanical properties of the finished crust leather. The mechanical properties, which included tensile strength, elongation ("stretchability"), Young's Modulus ("stiffness") and fracture energy ("energy required to open unit area of crack surface") were determined as per the procedures mentioned in previous article.[28] Subjective tests (break, handle, fullness and color) on the finished leather products were conducted by in-house (USDA) leather expert.

Microscopic Leather Surface Analysis

Finished leather panels produced from the control and alternatively cured hides (F-A to F-F) were analyzed under a stereo microscope to determine if there is any difference in the grain structure of the finished leather panels. Moreover, scanning electron microscope (SEM) images were examined to evaluate the impact of the new hide preserving formulations on the leather surfaces at microscopic level. The stereo microscope and SEM images were taken according to the published procedures.[28]

Results and Discussion

Five newly developed formulations were evaluated for their effectiveness in preserving bovine hides as well as for their environmental impact. The formulations were made by mixing 35% saturated brine solution (in one case 20% saturated brine solution) with different biocidal agents. These new formulations reduce salt consumption by about 60% in comparison to the traditional hide curing method. The efficacy of these alternative formulations in hide curing has been communicated in recently published article.[27] To address the pollution problem associated with the leather processing, an alternative method to conventional salt curing needs

to be developed. For the five alternative formulations, benzalkonium chloride (BAC), sodium benzoate (Na-B), 2-n-octyl-4-isothiazolin-3-one (OIT) and lactic acid (LA) have been used as biocides dissolved in low concentrated brine as described in Table I. To briefly describe some of the chemicals used in the formulations: sodium benzoate (Na-B) is an FDA approved food additive and is capable of stopping bacteria from reproducing. It also inhibits fungus growth.[35] BAC is a quaternary ammonium compound which is active against gram-positive and gram-negative bacteria and it is also used as a hide preservative.[36] 2-n-octyl-4-isothiazolin-3-one (OIT) is a biocide which has found application in paints, coatings, inks, household-cleaning products, building materials, plastics, textiles and wood treatment solutions.[37] OIT is water miscible and gets rapidly biodegraded to materials that are in turn readily biodegradable, therefore it is unlikely to persist in the environment. In addition, biological wastewater treatment can remove OIT from wastewater, so it has a low risk to be accumulated in the food chain and exhibits low toxicity to soil microorganisms, earthworms and birds.[38] Lactic acid is an FDA approved antimicrobial agent which is commonly used to control microbes in animal carcass and it is also known to be used as a humectant to attract moisture and enhance hydration of the skin, making it useful in reducing the roughness and scaling of the skin.[39,40,41] This research is conducted to overcome the pollution problem associated with the use of saturated brine as a hide curing solution. The new alternative hide curing formulations reduce the salt usage by 60% compared to conventionally used formulation. In this article, we have reported the impacts of using the newly developed hide preserving formulations on the quality of leather and on the environment. In terms of impact, also a comparison study between the alternative and traditional method of leather processing has been evaluated.

Effect of the Developed Formulations on Limiting Bacterial Growth

The effectiveness of the newly developed hide curing formulations on limiting bacterial growth was determined by counting the aerobic bacterial colony of the soaking liquor of the preserved hide samples after 35 days of storage. The bacterial count was quantified and expressed in log CFU/mL as shown in Figure 1. Hide sample treated with F-C was putrefied after 21 days of storage and removed from further investigation. Deterioration of the hide piece was characterized by discoloration, full hair slip and pungent odor. This may have resulted due to the insufficient amount of dehydrating agent (salt) used with F-C. In general, the bacterial counts for all other alternative formulations are lower than the control except for F-D. Especially, F-E and F-F showed the best efficacy in limiting the aerobic bacterial growth on hide surface throughout the storage time of 35 days. Soaking liquors for F-E and F-F count 2.92 and 6.38 log CFU/mL less than the control suggesting that the newly developed formulations are more effective to preserve hide from bacterial attack compared to the control during the storage period.

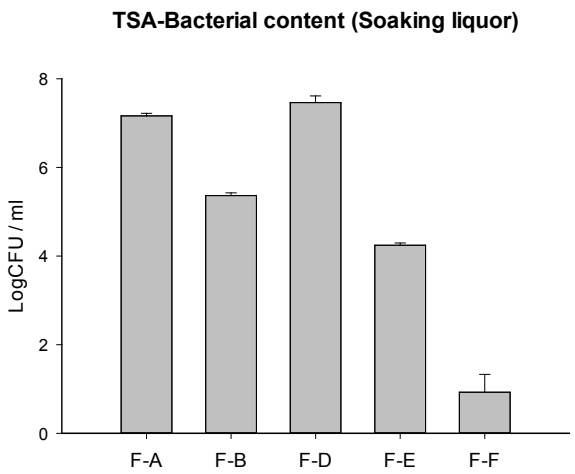


Figure 1. Total Aerobic Bacterial colony count of the soaking liquors from differently preserved hide samples

Effect of the Developed Formulations on Limiting Yeasts and Molds

All the newly developed formulations are found more effective compared to the control (F-A) in limiting yeast and mold growth according to the colony counts in soaking liquors (Figure 2). Yeast and mold recoveries from the soaking liquors are lessen by 4.21, 1.14, 3.88 and 2.64 log CFU/mL for F-B, F-D, F-E and F-F respectively in comparison to the control (F-A).

Determination of Chloride Content

The chloride content in soaking liquor directly reflects the salinity in tannery wastewater.

From Figure 3, it is clearly shown that, the amount of chloride content in soaking liquors from alternatively preserved hides is much less than that from conventionally cured hide (F-A). The chloride content reduction in soaking liquors resulted from the less usage of salt during hide preservation. In comparison to the control, F-D, F-E and F-F formulation treated hides generate 60, 52 and 26% less salt-waste respectively, in their soaking liquors. The variation in chloride content recovery was evolved mainly from the differences among the hide samples, although some of the saturated brine solution was used for curing. The more the

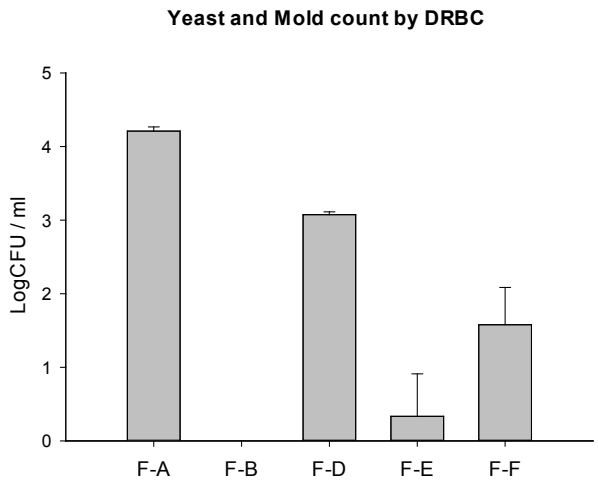


Figure 2. Yeast and molds colony counts of soaking liquors from differently preserved hide samples

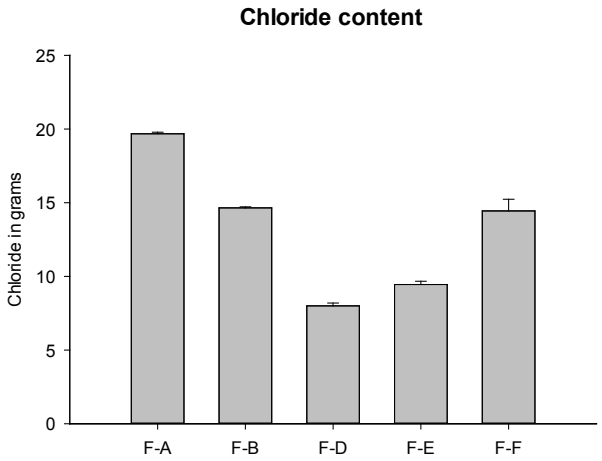


Figure 3. Chloride content (in gram) of used liquid produced from soaking 1 kg of preserved hide.

salt hide samples absorb during curing the more they will release during soaking. The salt absorbing capability of hide samples may vary for the presence of different amount of unexpected materials on internal hide surface, such as fats or associated meat even after using fleshed hide. However, the overall result demonstrates that, upon adoption, the alternative formulations will have much less post leather processing impact to the environment in comparison with the control.

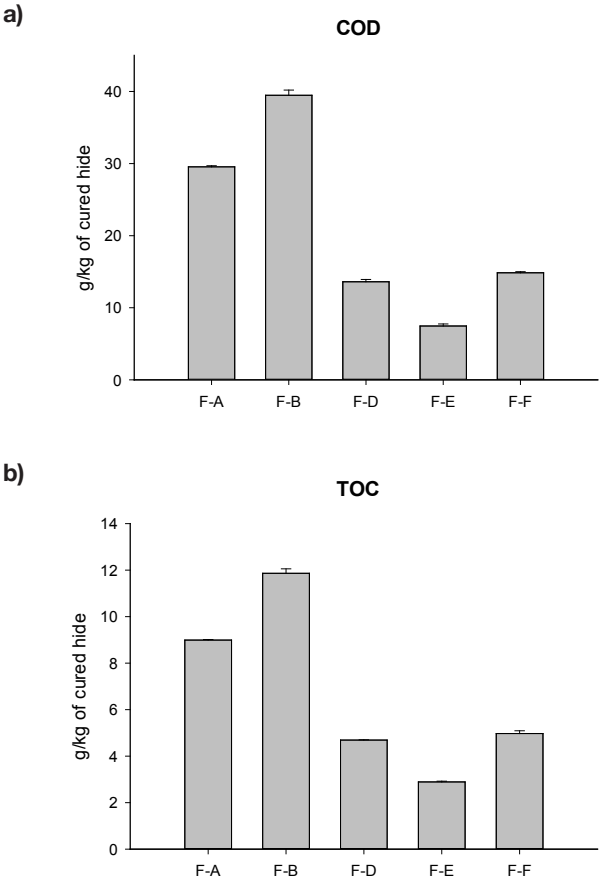


Figure 4. a) Chemical Oxygen Demand (COD) b) Total Organic Carbon (TOC) values of the soaking liquors of the hides preserved by each formulation. Both the values are expressed in g/Kg of cured hide.

Measurement of TOC and COD in Soaking Liquor

The COD and TOC results are found to be similar to each other with the highest value for F-B treated sample (Figure 4). For the other alternatively treated samples (F-D, F-E and F-F), COD and TOC values are significantly less than the control. The possible reason of having the highest TOC and COD values for F-B is that it contains the antimicrobial agents with the high number of carbon atoms compared to other antimicrobial agents used and therefore, a large amount of oxygen is needed to oxidize those carbons coming from the antimicrobial agents. On the other hand, the high COD and TOC values for the control (F-A) is related to the growth of bacteria, yeast and molds which is consistent to the results found in Figure 1 and 2. The presence of high level microorganisms during the hide preservation leads to degradation of proteins resulting in high carbon content in the soaking solution, which in turn requires large amounts of oxygen for the oxidation process.

Determination of BOD in Soaking Liquors

BOD analysis on the soaking solutions of the differently cured hides was carried out over a five-day period. As shown in Figure 5 the BOD load of the soaking liquor of the control is higher than the soaking liquors of alternatively cured hides. According to 5th day data, a 31 to 94% lower BOD load is recorded for the soaking liquors of alternatively preserved hides than the traditionally cured hide. The high BOD value for the control (F-A) could be because of the presence of high concentration of living microorganism (bacteria, yeast and mold) and also the subsequent microbial degradation of the hide. This is consistent with the bacterial, yeast and mold colony count results shown in Figure 1 and 2. The rapid increase of BOD value for F-A in the first 40 h also suggest the presence of high concentration of living microorganism in soaking liquor initially. In addition to the other biochemical substances, the dead cells of microorganisms could serve as a source of nutrients for the living cells present in the soaking liquor.

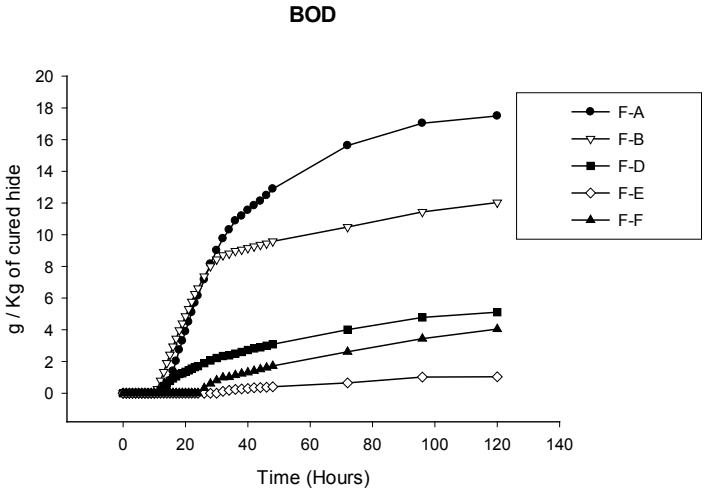


Figure 5. Biochemical Oxygen Demand (BOD) values of the soaking liquor measured over 5 days period.

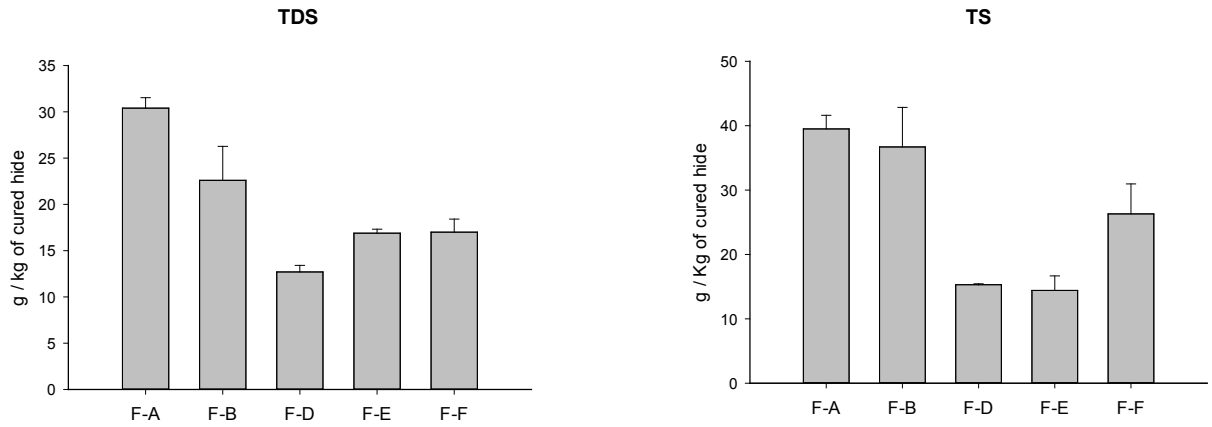


Figure 6. Solid pollutants generated from soaking process of 1 kg of differently cured hides: Total Solid (TS) and Total Dissolved Solid (TDS).

Determination of Solid Pollutants in Soaking Liquors

According to Figure 6, most of the soaking liquors from alternatively cured hides result in a significant decrease in both total solid (TS) and total dissolved solid (TDS) loads. Specially, F-D and F-E brought down the solid pollutants by more than 50% when compared to the control (F-A). This significant reduction in the TS and TDS values is because of the reduction in the amount of salt used for preservation with the newly developed formulations.

Quality Analysis of Crust Leather

Following the USDA standard tanning protocol, the hide pieces, preserved by the traditional and the newly developed formulations, were processed into crust upper shoe leather.

Grain Surface Analysis of Leather

The grain structure of each finished leathers was analyzed under a stereo microscope. The analysis was conducted to see the effect of each formulation on the surface fineness or coarseness of the crust leather. As shown in Figure 7, no noticeable difference was observed between the grain structure of leather produced from the traditionally cured hides and leathers produced from the alternatively cured hides. To further evaluate the surface feature of the crust leather a stereo microscopic image was captured at the crease upon folding the leather panels (Figure 8). Again, there was no noticeable difference between the leathers produced from the differently cured hides, indicating that none of the developed formulations caused harm to the hide grain.

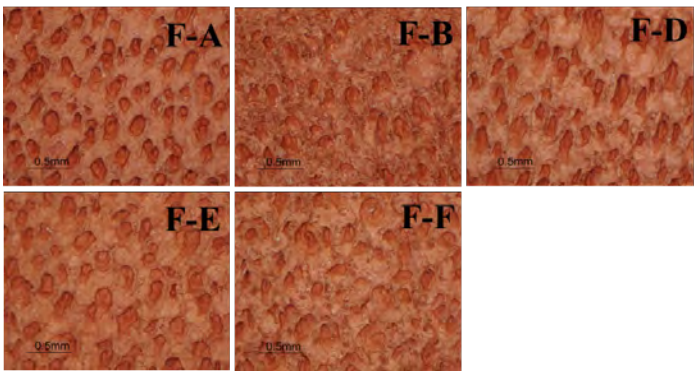


Figure 7. Stereo microscopic images of the crust shoe leather made from hides cured with different formulations.

Surface Analysis of Crust Leather using Scanning Electron Microscope

Surfaces of the crust leather produced from the traditionally and alternatively preserved hides were analyzed using SEM at 100 x magnification. As shown in Figure 9, there was no significant difference

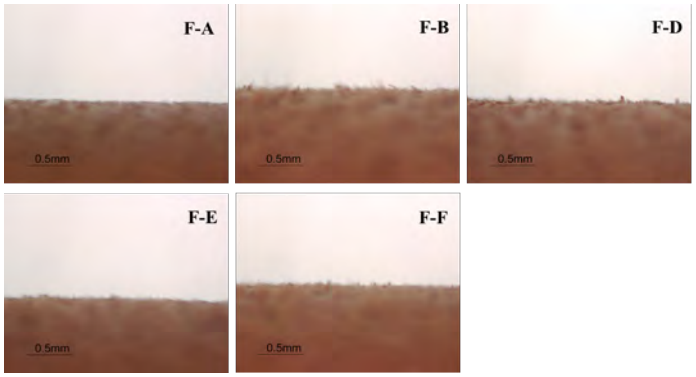


Figure 8. Stereo microscopic images of the leathers at the crease

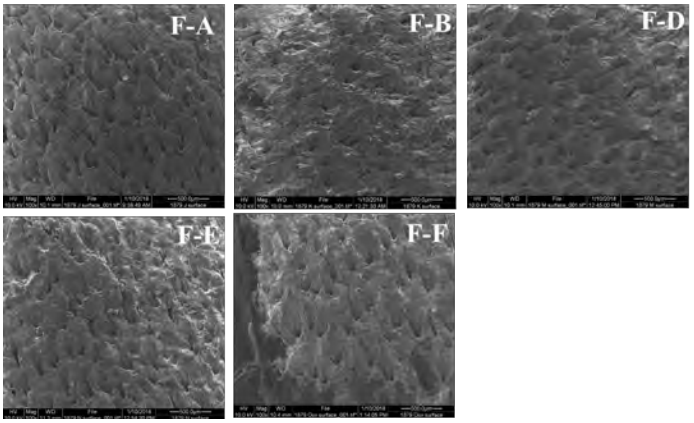


Figure 9. SEM surface images of crust leathers from the differently preserved hides

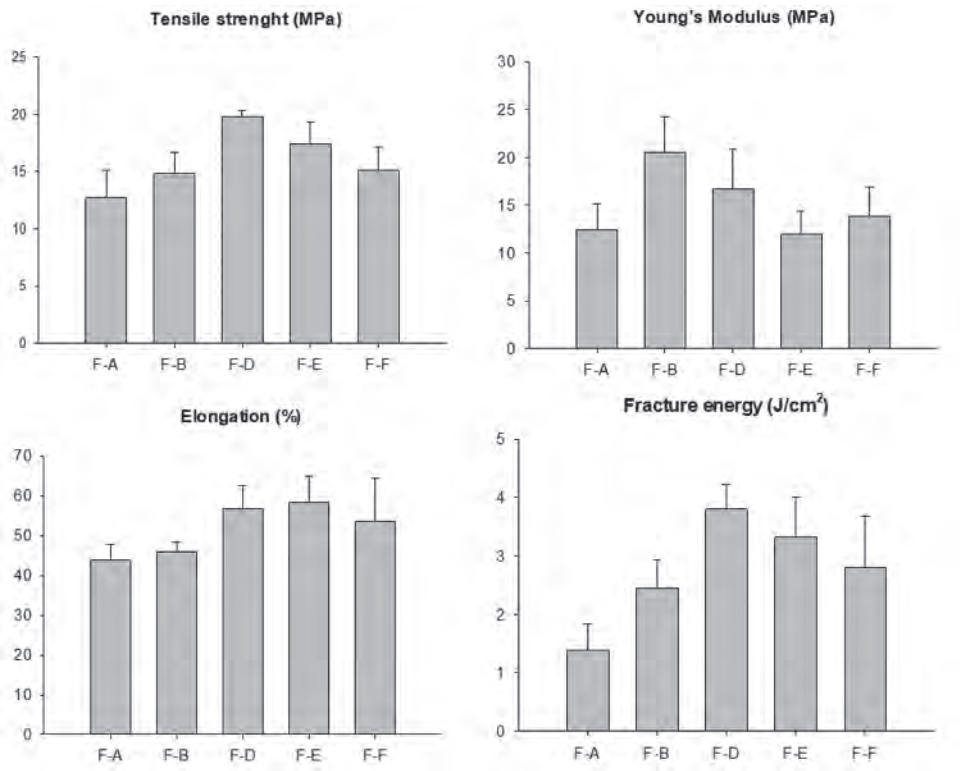


Figure 10. Mechanical properties of the finished leathers made from hides cured with the different formulations

observed when the experimental samples (F-B to F-F) were compared with the control (F-A). Therefore, no detrimental impact from the newly developed hide curing formulations on finished leather.

Determination of Mechanical Properties of Leather

From Figure 10, it is evident that the new hide preserving formulations do not have any adverse effect on the finished leather in terms of property analysis (Tensile Strength, Young's Modulus, Fracture Energy and Elongation). The overall mechanical properties of the leather produced from the experimentally preserved hides were found comparable or even better than that produced from the control. For instance, leather produced from F-E cured hide exhibited improved property in every parameter as shown in Figure 10 in comparison to the quality of leather yielded from traditionally preserved hide sample (F-A).

Subjective Test Analysis of Leather

In-house expert on leather assessed the crust shoe leather panels for their fullness, softness, color, grain tightness (break) and general appearance by hand and visual examination (Figure 11). The subjective test results were expressed on a scale of 0-5 points with 0 being the worst and 5 being the best subjective test outcome.

The leathers produced from the experimentally cured samples displayed similar fullness, grain, handle, color and general appearance. Overall, the alternatively preserved hides produced leathers of similar quality to the traditionally processed leather (F-A), indicating that the biocides utilized with the new formulations do not damage the hides.

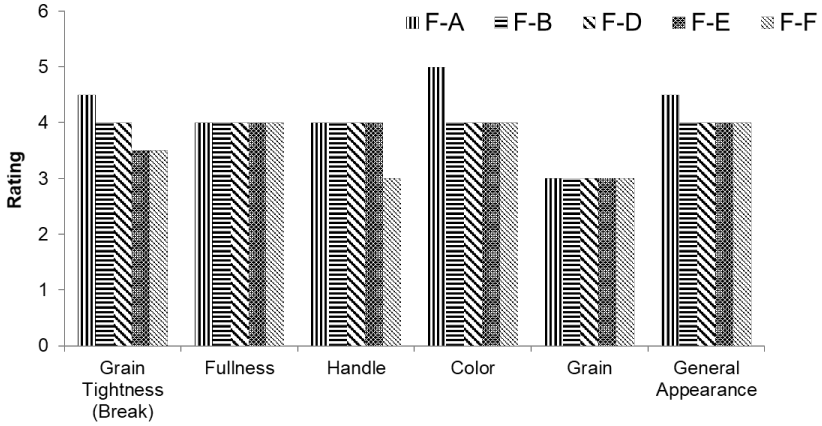


Figure 11. Organoleptic evaluation of crust leathers from differently cured hides

Conclusion

The inventive formulations have demonstrated to be effective in preserving bovine hides by preventing bacterial growth on the hides better than the traditional formulation (95% saturated salt solution) for 35 days or more. The new formulations utilize around 35% saturated brine solution, which is around 60% reduction in salt usage compared to conventional formulation. While the dehydrating brine of the formulation keeps the moisture level of the preserved hide low creating inconvenient condition for the microbes to grow, the low concentration antimicrobial agents inhibits or kills the bacteria simultaneously. The environmental impact studies reveal that pollution loads of the process discharge from the alternatively cured hides are significantly lower than the traditionally cured hide as demonstrated by the measured values of chloride content, TOC, COD, BOD, TDS. From the SEM surface images, mechanical properties, grain pattern analysis and organoleptic evaluation, the newly developed formulations do not appear to have any negative effect on the finished crust leather. The results of all the experiments conducted in this study suggest that, these new formulations could be considered as viable alternatives to the conventional salt curing formulation.

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The Quality of Leather Estimated from Airborne Ultrasonic Testing of Hides

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Abstract

High-quality hides are paramount for competitiveness in both domestic and export markets. Currently, hides are visually inspected and ranked for quality and sale price, which is not always reliable when hair is present on the hides. Advanced technologies are needed to nondestructively and accurately characterize the quality of hides and enable one to estimate the qualitative and mechanical properties of leather. We were the first to carry out research for airborne ultrasonic (AU) methods to nondestructively characterize the quality of hides. The developed nondestructive method is based on measuring the AU waves transmitted through the hide samples. Research results demonstrated that the average amplitude distribution received from the ultrasonic wave transmitted through the hide samples yielded the best correlation with the AU test variables: gain, speed, and frequency. Observations showed AU parameters derived from the average distribution values for amplitude (AMPa) and time of flight (TOFa) have a correlation with the quality of leather. This study demonstrated that the fullness, overall characteristic, tensile strength, stiffness, elongation, and toughness of leather could be nondestructively estimated by the ultrasonic quantities obtained from AU testing of hides.

Introduction

Cattle hides are the highest value byproduct of the beef industry. High quality, clean, and well-preserved hides are important for competitiveness in both domestic and export markets. Hides currently, however, are visually inspected and ranked for quality and sale price. Hides cannot be effectively sorted at the earliest stage of processing, because when hair is present, the visual inspection is not reliable for evaluating the quality of hides. Development of an objective and nondestructive method to accurately characterize the quality of hides is necessary and indispensable. Efforts have been made to develop new technologies to provide a quality estimation prior to the leather making process.[1-6]

It has been reported that with appropriate Airborne Ultrasonic (AU) equipment and methods, it is possible to perform sensitive inspections for defects such as voids, cracks, and disbonds in a wide variety of products such as lumber and composites.[7-12] The optimization for resolution or penetration can be achieved by selecting a correct range of AU frequencies for testing. The timber industry was among the first that employed AU commercially. Applications include: (1) inspection of internal decay, voids, and cracking (2) detection of delamination and cracking in composite or processed wood products, and (3) assessment of wood quality by measuring sound transmission velocity. In addition, composite materials, particularly within the aerospace industry, have been a primary area of application for AU methods.[8,13] We were the first to develop AU methods to detect leather defects and characterize its quality. In the previous reports, we presented that AU testing without direct contact with samples offers a great potential for the nondestructive evaluation of the quality of leather.[1-2] As a non-contact technique, AU has shown great potential to be an ideal characterization method for large leather or hides.

The AU sensor is designed for dynamic measurements and offers several key advantages in automation applications. In general, airborne ultrasonic testing involves pulsing ultrasonic signals at the material and measuring the reflected amplitude of those signals emanating from the material. The amplitude of ultrasonic signals reflected at the surface of a planar material (such as films, sheets, fabrics, and leather or hides) is a function of the material's surface morphological variations. Therefore defects, such as scars, insect bites, or knife cuts should be able to be detected because they will change the intensity of the AU signal reflecting from the surface of the material. Our studies indicated that AU testing could reveal the presence of defects or other physical discontinuity in leather, which could affect the leather quality.[2-4] Leather quality such as tensile strength and stiffness showed a good correlation with the AU data, implying those properties could be estimated from AU testing.

Our current AU testing involves pulsing ultrasonic waves and measuring the amplitude of those waves transmitted through the

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material.[3-6] By using the through-transmission mode, we observed more useful information can be extracted from the AU scan than the reflective mode, particularly for hides which are covered by hair.

Our previous studies indicated that the key for success in AU testing for soft materials such as hides and leather is to use AU transducers with low frequencies, which leads to effective transmission of ultrasonic waves through the samples.[3-5,14] The variations in the AU quantities, such as amplitude (AMP) and time of flight (TOF) were color coded into C-scan images to reveal the location and shape of the defects or some other physical discontinuity existing in the samples. The research was recently carried out to optimize AU methods for hides and leather inspections and to study the effects of transducer frequency, thickness of leather and AU gain on the resultant AU amplitude received, which was then color-coded into a C-scan image.[5] Observations showed the 100 kHz transducer works well for crust leather. This study also showed the AMP and TOF are strongly affected by the sample thickness and instrument gain applied to the AU tests. Moreover, in a recent experiment, we found that the distribution of Time of Flight (TOF) is a better AU parameter to correlate the mechanical properties of leather.[6] Observations showed tensile strength, stiffness, fracture energy, and grain break of leather could be measured nondestructively by using the AU methods described in an earlier report.[6] These results will be instrumental in finalizing the development of AU technology for characterizing the quality of hides.

In this new study, we have identified the proper parameters for AU testing of raw hides. A statistical experimental design was used to establish the relationship between key test parameters (instrument gain, speed of scanning and ultrasound frequency) and corresponding AU quantities thereby identifying appropriate AU testing methods for hides. We also investigated the relationship between AU quantities of hides, the qualitative and mechanical properties of leather from the AU tested hides. We discovered that when two AU physical quantities were used jointly, they showed a good correlation with the quality and property of leather tanned from the respective hides.

Theory

A large portion of the energy is reflected, and the remaining energy is transmitted, as an ultrasound wave is passed from one medium to another (for example from air to hides). The key parameter that governs the reflected and transmitted relationship is referred to as acoustic impedance (Z) = $(\rho E)^{1/2}$, where ρ = density of medium, E = elastic constant of medium.[15] This square-root relationship shows the acoustic impedance of a material and is governed by the elasticity and density of a material. If one knows the acoustic impedances of the materials (media 1 and 2) on both sides of the boundary, the fraction of the incident wave intensity reflected can be calculated as the acoustic reflection coefficient (R), $R = (Z_1-Z_2)^2 / (Z_1+Z_2)^2$, where: Z_1 = acoustic impedance of medium 1 and Z_2 = acoustic impedance of medium 2.[16] The greater the R value indicates the higher the percentage of energy will be reflected at the interface or boundary between one medium and another. Therefore, it can be expected that the mismatch of acoustic impedance weakens the sound wave transmission. In addition, defects such as voids, insect damage, and brands will change the acoustic reflection coefficient and consequently affect the amplitude of the wave transmitted through the material which will show up in AU images such as either AMP or TOF C-scans.

Experimental

Materials and Methods

Fleshed cattle hides were collected from JBS (Souderton, PA). Twenty-three approximately 30 cm x 30 cm pieces were cut out evenly among the two sides of a heavy steer hide. All hide pieces were sealed in plastic zip-lock bags and stored in a freezer. Before the test, they were transferred to a refrigerator to thaw out overnight. Then the hides were clamped vertically using large binder clips across two parallel bars to minimize any slack in the sample. The hair was left untouched and intact to simulate real life conditions. Scanning was performed on the hides using an AU system. When performing AU testing, AU waves must travel from air, which is a medium with low acoustic impedance, to a medium such as hides and leather with considerably higher acoustic impedance. Therefore selection of the proper AU transducers and frequency are critical to achieving enough penetration of ultrasonic waves in order to extract important information related to the structure and properties of leather such as the number of defects, morphology, strength and softness. Figure 1 shows a photo of an AU testing system which consists of two ultrasonic transducers, a transmitter with a diameter of 38 mm pulsed with a tone burst through a power amplifier (model: NCG50-D38, The Ultran Group, State College, PA), and a receiver (model: NCG50-D38) with the same size as the transmitter connected to a preamplifier. All these components were mounted on a computer-controlled 3-D scanner (UPK-T24, MISTRAS Group,

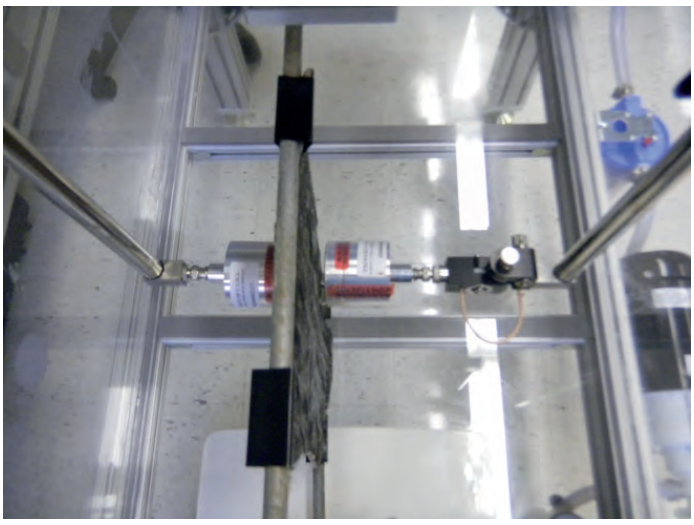

Figure 1. AU testing system

Inc., Princeton Jct., NJ). The software UTWIN (version E1.81, also from MISTRAS Group, Inc.) was used to control the movement of transducer/receiver array over the entire surface of the hide for conducting a C-scan.

There are various AU quantities that can be displayed as a function of time or position for one AU scanning result. The velocity, amplitude (AMP) and duration of ultrasonic waves (Time of Flight, TOF) measured by the receiver and changed with the material properties of the samples. The C-scan is very commonly used in AU testing in which the transmitted AU pulses are captured and the amplitudes of the transmitted pulses are mapped using pseudo color from the maximum amplitude in gate 1 or gate 2 set on the A-scan.[6] The A-scan presents the waveform of the received signal and gate 1 is set to the first waveform and gate 2 is set to the second waveform. C-scan is an automated two-dimensional scanning system in AU testing which provides a planar-type view of the location and size of the test specimen. The resolution of the C-scans generated was set to 0.5 mm in both the scanning and indexing directions. The amplitude and TOF of the transmitted signals at regular intervals were recorded, color-coded and mapped into an image file as shown in Figure 2. The proper conversion of C-scan images to numeric data is a critical step to realize the quantitative representation of hides. Based on our previous research,[4,6] gate 1 representing the direct transmission through the material and gate 2 showing the thickness reflection of waveform bouncing through the sample were set to collect data at designed intervals. All the data recorded in the UTWIN software under designed testing conditions were screened to eliminate the data set containing null values. The effective data were further analyzed through the statistical software Minitab-19 (Minitab LLC, State College, PA).

Experimental Design

The Box and Hunter's central composite rotational design was used to establish the relationship between variables and responses.[17-18] Three important factors of AU testing including gain (X_1), speed (X_2) and frequency (X_3) were studied for their effects on the resultant AU physical quantities (such as AMP and TOF). In order to simplify the regression equations, the variables X_i were coded from original values (X_i'). The coded values of X_1, X_2 and X_3 were obtained by the following equations: $X_1 = (X_1'-10)/10$, $X_2 = (X_2'-30)/10$, $X_3 = (X_3'-50)/20$. All the experimental conditions and coded values were listed in Table 1, including 9 center points. Based on different responses under testing conditions, regression models were calculated containing variables presented as their linear, quadratic and bifactorial cross products. Meanwhile, statistical analyses were performed to identify the strongest relationship between variables and responses.

After AU measurement, the hide pieces were taken off the clamps and stamped using a custom-made number die (Durable Mecco, Franklin Park, IL) with the size of the numbers being 1.27 cm x 1.27 cm. Five numbers were hand-stamped into the hides with 4 of the numbers in a square pattern approximately 6 cm apart from the center, and 1 number stamped directly in the middle. The stamped hide pieces (named marked hides hereafter) were placed back on the clamps and retested using the same conditions. After AU testing, the hide pieces were placed back into the freezer until all of the hide pieces were tested according to the experimental plan as shown in Table I. The hides pieces were then tanned and retanned using the methods reported previously.[19-20]

Table I

The design of experimental conditions

Run	Coded values			Actual values		
	X_1 Gain	X_2 Speed	X_3 Frequency	X_1' Gain (dB)	X_2' Speed (mm/min)	X_3' Frequency (kHz)
1	-1	-1	-1	0	20	30
2	-1	-1	1	0	20	70
3	-1	1	-1	0	40	30
4	-1	1	1	0	40	70
5	1	-1	-1	20	20	30
6	1	-1	1	20	20	70
7	1	1	-1	20	40	30
8	1	1	1	20	40	70
9	-1.682	0	0	-6.82	30	50
10	1.682	0	0	26.82	30	50
11	0	-1.682	0	10	13.18	50
12	0	1.682	0	10	46.82	50
13	0	0	-1.682	10	30	16.36
14	0	0	1.682	10	30	83.64
15	0	0	0	10	30	50
16	0	0	0	10	30	50
17	0	0	0	10	30	50
18	0	0	0	10	30	50
19	0	0	0	10	30	50
20	0	0	0	10	30	50
21	0	0	0	10	30	50
22	0	0	0	10	30	50
23	0	0	0	10	30	50

Quality Assessments

The samples were rewet and staked twice before being evaluated by an experienced tanning professional for the qualitative leather properties: handle, fullness, grain (break), color and overall characteristic. Among them, handle is the sensation or judgment by feeling for certain physical properties of leather through touch with the fingers and hands for the flexibility and smoothness of the leather. Fullness is the feeling of compressed leather in the hand. A full leather fills the palm with the force to bounce back, while a flat leather has more of a cardboard effect. Grain break is the way to characterize the wrinkles formed on the surface of leather when

bent grain inward. In general, the finer the wrinkles the better the quality of the tested leather. A scoring system from 1 to 5 was used for each property, where 1 is the worst while 5 is the best. Based on the scores of these four properties, an overall characteristic was also determined and reported on a scale of 1 to 5. In addition, the relationship between AU quantities on hides and subjective scores from the leathers was explored.

Mechanical Property Tests

Leather samples were also tested for mechanical properties. An Insight-5 test frame and Testworks-4 data acquisition software (MTS Systems Corp., Minneapolis, MN) were used throughout this work. Tensile strength, Young's modulus, elongation and fracture energy (toughness) were tested as described in a previous paper.[6] The samples were cut parallel to the backbone with 1x 10 cm in shape. Tested at 25.4 cm/min with a 5 cm grip or gage length. All samples were preconditioned in an environmental chamber at 23 ± 2°C and 50 ± 4 % RH for at least 24 h (ASTM D1610).

Results and discussion

C-scan images of unmarked hides (Figure 2a, Figure 2c) and marked hides (Figure 2b, Figure 2d) are demonstrated in Figure 2. AMP average distribution values (AMPa) were recorded in the UTWIN software, which represent the percentage of waves that reached the maximum amplitude from gate 1. The TOF average distribution values (TOFa) were recorded also from gate1. The average distribution values (AMPa and TOFa) and other AU data were calculated by the UTWIN software.

Through analyzing the response surface design by Minitab, a full quadratic regression equation was obtained with corresponding analytical values. First, R-squared values were compared among all models. R-squared is a statistical measure of how close the data are to the fitted regression line. After the examination of various AU quantities, the average amplitude (AMPa) showed to be the most sensitive response to the change in the three important test variables (gain X_1, speed X_2, and frequency X_3). The R-squared for the derived quadratic model is 0.97 (Table II) which is the highest among all other equations.

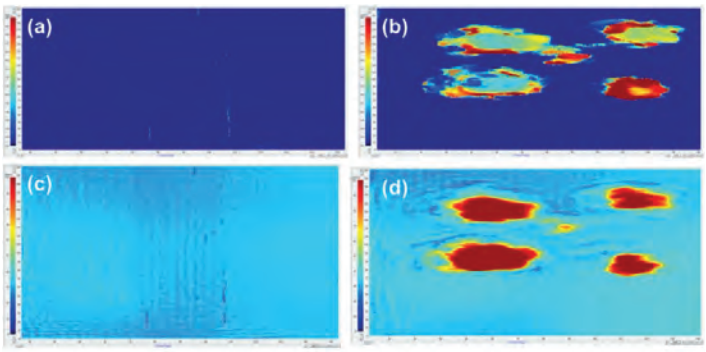

Figure 2. C-scan images of AMP for (a) non-marked hide, (b) marked and C-scan images of TOF for (c) non-marked hide, (d) marked hide

Table II

R-squared values of established models

Model	R-sq	R-sq(adj)	R-sq(pred)
Full quadratic model	97.28%	95.41%	83.21%
Adjusted model	97.16%	96.53%	94.35%

*R-Sq (adj): Adjusted R-squared; R-sq(pred): Predicted R-squared

The corresponding regression model is expressed as follows:

$$Y=27.77+26.32X_1-0.04X_2-19.47X_3+8.46X_1^2-0.97X_2^2-0.14X_3^2-0.75X_1X_2-16.4X_1X_2-0.63X_2X_3 \quad (1)$$

In general, the higher the R-squared, the better the model fits the data. However, R-squared may not reflect the whole story, and there are other analytical values that need to be considered for a good fit. As seen in the analysis of variable table (Table III), the p-value of "lack of fit" is 0.017, which rejects the null hypothesis that the model fits the data well at a significance level (α) of 0.05. In other words, one may conclude that the model does not adequately reflect the whole picture of data. Based on p-values, some terms in the equation are not meaningful since their high p-values (>0.05) indicate the acceptance of the null hypothesis that the coefficient has no effect on the model equation. For example the p-value of speed is 0.981. It is strong evidence that as a variable, speed may not be a significant

Table III

Analysis of variance of the full quadratic model

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	17955.1	1995.01	51.76	0.000
Linear	3	14640.0	4880.01	126.60	0.000
Gain	1	9460.5	9460.50	245.43	0.000
Speed	1	0.0	0.02	0.00	0.981
Frequency	1	5179.5	5179.52	134.37	0.000
Square	3	1156.2	385.41	10.00	0.001
Gain*Gain	1	1139.0	1138.98	29.55	0.000
Speed*Speed	1	14.8	14.81	0.38	0.546
Frequency*Frequency	1	0.3	0.29	0.01	0.932
2-Way Interaction	3	2158.8	719.60	18.67	0.000
Gain*Speed	1	4.5	4.45	0.12	0.739
Gain*Frequency	1	2151.2	2151.22	55.81	0.000
Speed*Frequency	1	3.1	3.14	0.08	0.780
Error	13	501.1	38.55		
Lack-of-Fit	5	389.2	77.85	5.57	0.017
Pure Error	8	111.9	13.98		
Total	22	18456.2			

*DF: Degree of freedom; Adj SS: Adjusted sums of squares; Adj MS: Adjusted mean squares

Table IV

Analysis of variance of the adjusted model

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	4	17932.4	4483.10	154.06	0.000
Linear	2	14640.0	7320.01	251.55	0.000
Gain	1	9460.5	9460.50	325.11	0.000
Frequency	1	5179.5	5179.52	177.99	0.000
Square	1	1141.2	1141.16	39.22	0.000
Gain*Gain	1	1141.2	1141.16	39.22	0.000
2-Way Interaction	1	2151.2	2151.22	73.93	0.000
Gain*Frequency	1	2151.2	2151.22	73.93	0.000
Error	18	523.8	29.10		
Lack-of-Fit	10	411.9	41.19	2.95	0.070
Pure Error	8	111.9	13.98		
Total	22	18456.2			

*DF: Degree of freedom; Adj SS: Adjusted sums of squares;
Adj MS: Adjusted mean squares

factor in the equation. Therefore, a more concise equation (equation 2) was obtained after removing insignificant terms. Its corresponding variance analysis was shown in Table IV. From the table, we could find that the p-value of Lack of Fit is 0.07 which is larger than the significance level (α) of 0.05, so it is possible to conclude that this adjusted model fits the data well. Additionally, both adjusted R-squared and predicted R-squared (Table II) improved compared with the full quadratic model.

$$Y=27.77 + 26.32\ X_1 - 19.47\ X_3 + 8.46\ X_1^2 - 16.4\ X_1X_2 \qquad (2)$$

According to Equation 2, a 3-D plot of the response surface of amplitude average distribution (AMPa) value as a function of speed and gain was shown in Figure 3 (a). It is demonstrated that AMPa has a significant increase with the increase of gain. Moreover, Figure 3 (b) shows a 3-D plot of the response surface of AMPa as a function of AU frequency and speed. We notice there is a negative correlation between AMPa and frequency. With the reduction of frequency during the test the average amplitude dramatically increases. From these two plots the effects of speed on amplitude are very limited,

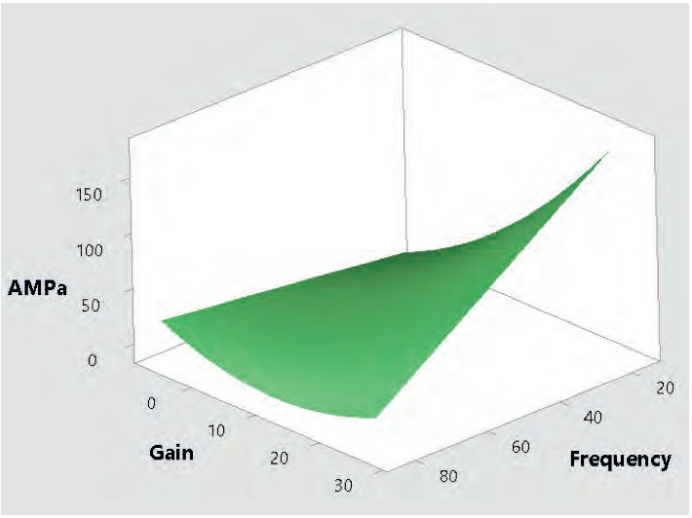

Figure 4. 3-D regression plot of AMPa as a function of gain and frequency

which confirms the statistical analysis that speed is not a meaningful term in the equation.

Moreover, the relationship among AMPa, frequency and gain is shown in Figure 4. It is evident that there is no linear increase or decrease trend between each factor and response. Frequency and gain may interact with each other to affect the response.

After determining the condition to obtain the most sensitive response under testing variables we also explored the relationship between AU physical quantities measured from hides and subjective qualitative evaluation scores of the corresponding leather after tanning. In this study, we have used the Pearson correlation to examine the strength and direction of the linear relationship between each pair of variables. The highest Pearson correlation coefficient for AMPa was found with fullness, which is 0.66. It represents a positive relationship between them. In other words, when the AMPa increases the fullness score also increases. The fullness of leather is closely related to its inner structure. The fiber bundles remain intact and well-distributed throughout the structure in the leather and retain their elasticity which may also contribute to the easier transmission of ultrasonic waves. Therefore, waves with higher amplitude were collected by the receiver. The p-value is 0.053,

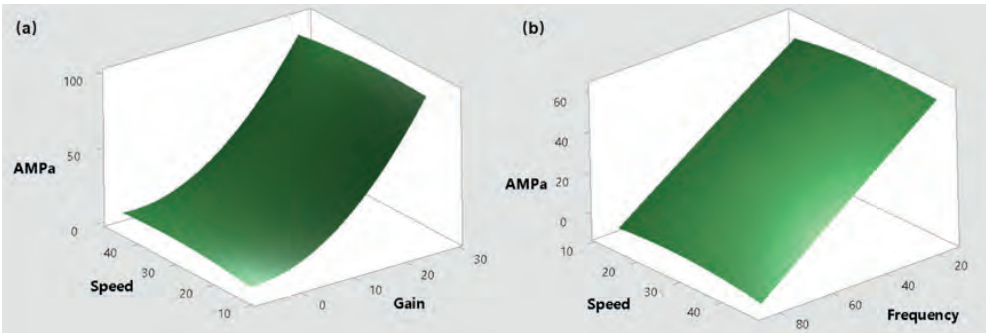

Figure 3. 3-D regression plot of AMPa as a function of (a) gain and speed; (b) frequency and speed

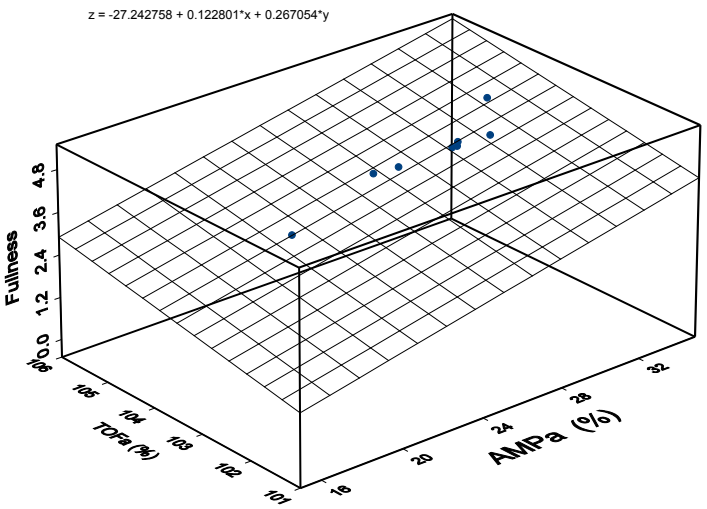

Figure 5. Fullness as a function of TOFa and AMPa

Table V

Pearson correlation matrix among subjective scores and AMPa

	AMPa	Break	Handle	Fullness	Color
Break	*0.099				
	**0.800				
Handle	0.559	0.500			
	0.118	0.170			
Fullness	0.660	0.300	0.750		
	0.053	0.433	0.020		
Color	0.388	0.550	0.500	0.750	
	0.303	0.125	0.170	0.020	
Overall	0.181	0.575	0.812	0.750	0.688
	0.642	0.105	0.008	0.020	0.041

*Pearson correlation, ** P-Value

which is close to the significance level of 0.05. This indicates that the correlation between AMPa and fullness is more significant than other pairs. Moreover, as shown in Figure 5, we have demonstrated there is a good correlation between the degree of fullness and the combination of AMPa and TOFa. On the other hand, in Figure 6, the overall characteristic of leather showed a similar trend as fullness, which implied that fullness is a key factor affecting the value of overall characteristic of leather.

Along with the AMPa, the relationship between different subjective evaluation categories was also assessed in Table V. The correlation coefficient between handle and overall is 0.812, and the p-value is 0.008. It is very obvious that a strong positive correlation exists between handle and overall. The overall characteristic of the leather was determined by combining all of the other subjective qualitative values such as fullness, grain break, color distribution and handle. It is possible to use only the handle score to evaluate the overall quality of leather since it can almost reflect the whole picture.

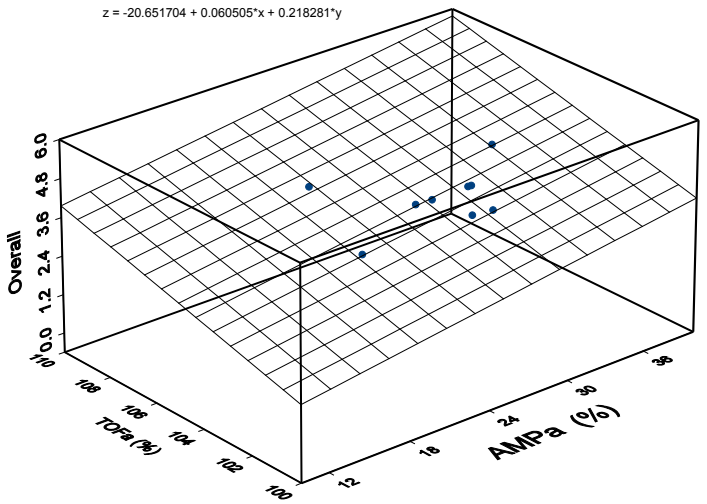

Figure 6. Overall characteristic as a function of TOFa and AMPa

This research also investigated the relationship between AU quantities measured from hides and the mechanical properties of corresponding leather tanned from the same hides. Tensile strength determines the maximum tensile stress the leather can sustain without fracture. Adequate tensile strength is very important in manufacturing leather goods where the leather is often subjected to a tensile force during mechanical stretching or elongation. Moreover, in a variety of end uses, leather goods must be capable of resisting considerable stress without fracture. Figure 7 displays a 3-D regression plot of tensile strength as a function of AMPa and TOFa simultaneously, where the dots are data points. Tensile strength shows a strong positive correlation with TOFa, while it has only a small negative correlation with AMPa. The reason for this difference is related to the leather structural difference. TOFa is a distribution of time of flight which is increased with the transit time of the sound waves through the hide sample. It reflects how "smooth" the sound can travel through the hide sample, which is

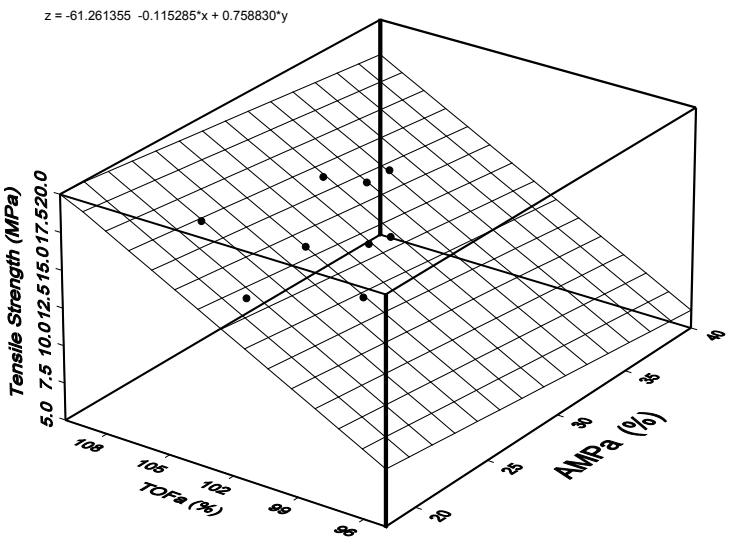

Figure 7. Tensile strength as a function of AMPa and TOFa.

governed by the internal structure of the material. The greater amount of TOF reflects the denser fibrous structure of hides, which results in a longer transmission of ultrasonic waves through the hides therefore it could indicate higher tensile strength values for leather. However, this phenomena may not be applicable to the AMPa, probably because the AMP is more related to the elasticity of the hide fibers. The higher AMP indicates higher rigidity, which has a negative impact on the tensile strength of leather. Importantly, the results demonstrated in Figure 4 imply that the tensile strength of leather could be estimated by the AU nondestructive test method described here by measuring the TOF of the original hides.

Stiffness is one of the most important physical quantities characterizing the mechanical properties of leather. It expresses the resistance of leather subjected to a small tensile deformation. It is commonly known that the higher Young's modulus, the stiffer the material. In the literature, its reciprocal has been named compliance.[21] In various reports, Young's modulus has been linked to the fine structure of leather, such as the degree of fiber orientation[22-23] and fiber adhesion.[24] It also has been associated with

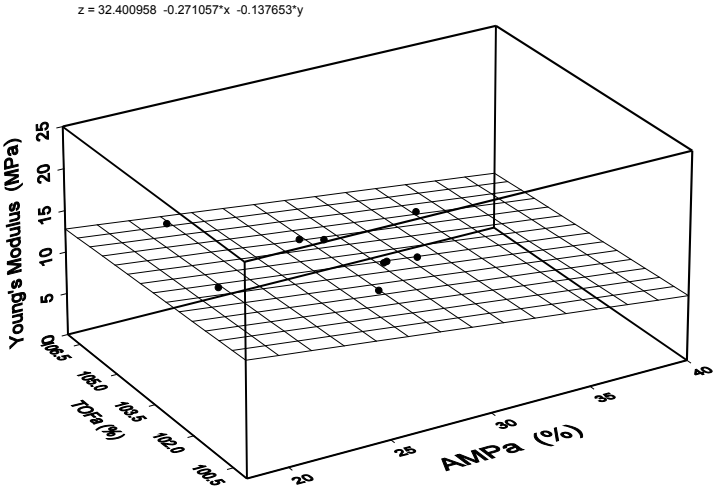

Figure 8. Young's modulus as a function of AMPa and TOFa

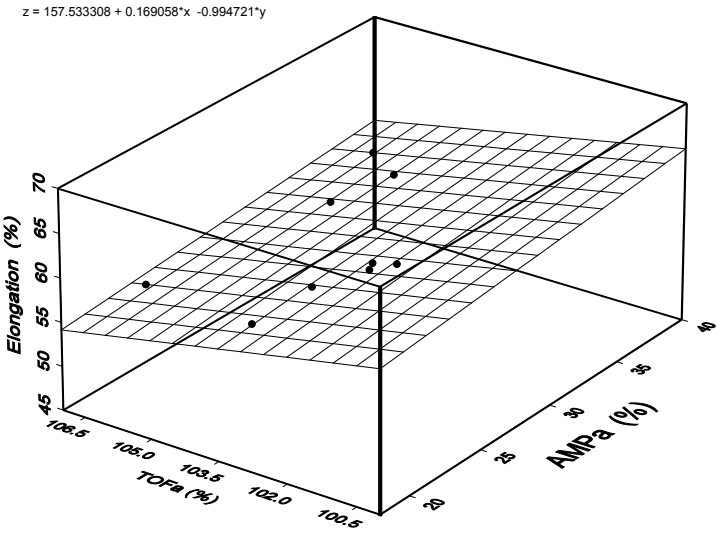

Figure 9. Elongation as a function of AMPa and TOFa

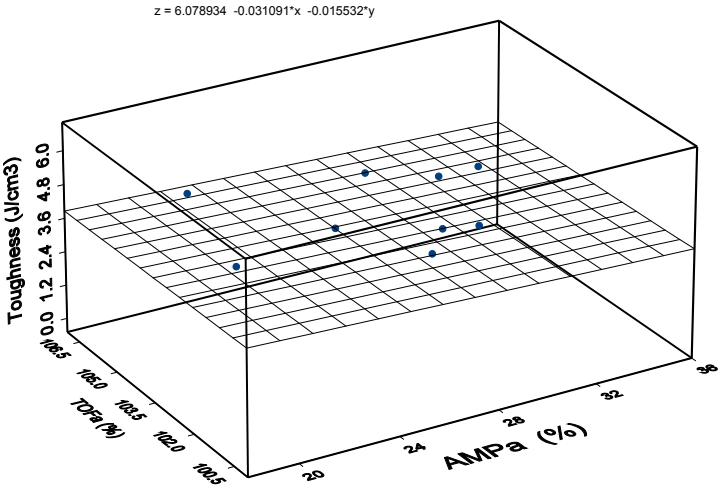

Figure 10. Toughness as a function of AMPa and TOFa.

leather softness, temper and handle in various reports.[25-27] Figure 8 is a regression plot of Young's modulus as a function of AMPa and TOFa. It shows the Young's modulus negatively correlates with AMPa but has little effect on TOFa.

A 3-D regression plot of elongation as a function of AMPa and TOFa is shown in Figure 9, where the elongation shows little change with AMPa and however decreases significantly with TOFa. Again, this behavior is probably due to the denser fiber structure which is more resistant to an extension when it is subjected to a tensile force.

Toughness (also known as fracture energy in materials science) was determined by measuring the energy required to fracture the leather sample which is the area under the stress-strain curve.[28] As we have reported previously, contrary to tensile strength, the measurement angle shows little effect on the toughness.[28] Good toughness is an important quality requirement for leather or other fibrous materials, which reflects a superior balance of strength and flexibility. Figure 10 displays a 3-D regression plot of toughness as a function of AMPa and TOFa, in which both AU quantities show little effects on toughness. This could be due to the structural factors affecting toughness balanced out in the AMPa and TOFa measurement. Therefore these two AU parameters do not affect fracture energy significantly.

Conclusions

The objective of this research is to develop a nondestructive method using AU to characterize the quality of hides and further to estimate the qualitative and physical properties of leather. This method is based on measuring the AU quantities as ultrasound waves are transmitted through the hide samples. In this investigation, we discovered the amplitude average distribution value (AMPa) yielded the best correlation with the AU testing variables. Observation showed that the fullness, overall characteristic, tensile strength, stiffness, elongation and toughness of leather could be estimated by

the AMPa and TOFa quantities tested from corresponding hides. The results derived from this research are instrumental in establishing a quality control/quality assurance method for manufacturing.

Acknowledgement

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Lifelines

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Cheng-Kung Liu, Ph.D. see *JALCA* **94**, 158, 1999

Nicholas P. Latona see *JALCA* **96**, 401, 2001

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