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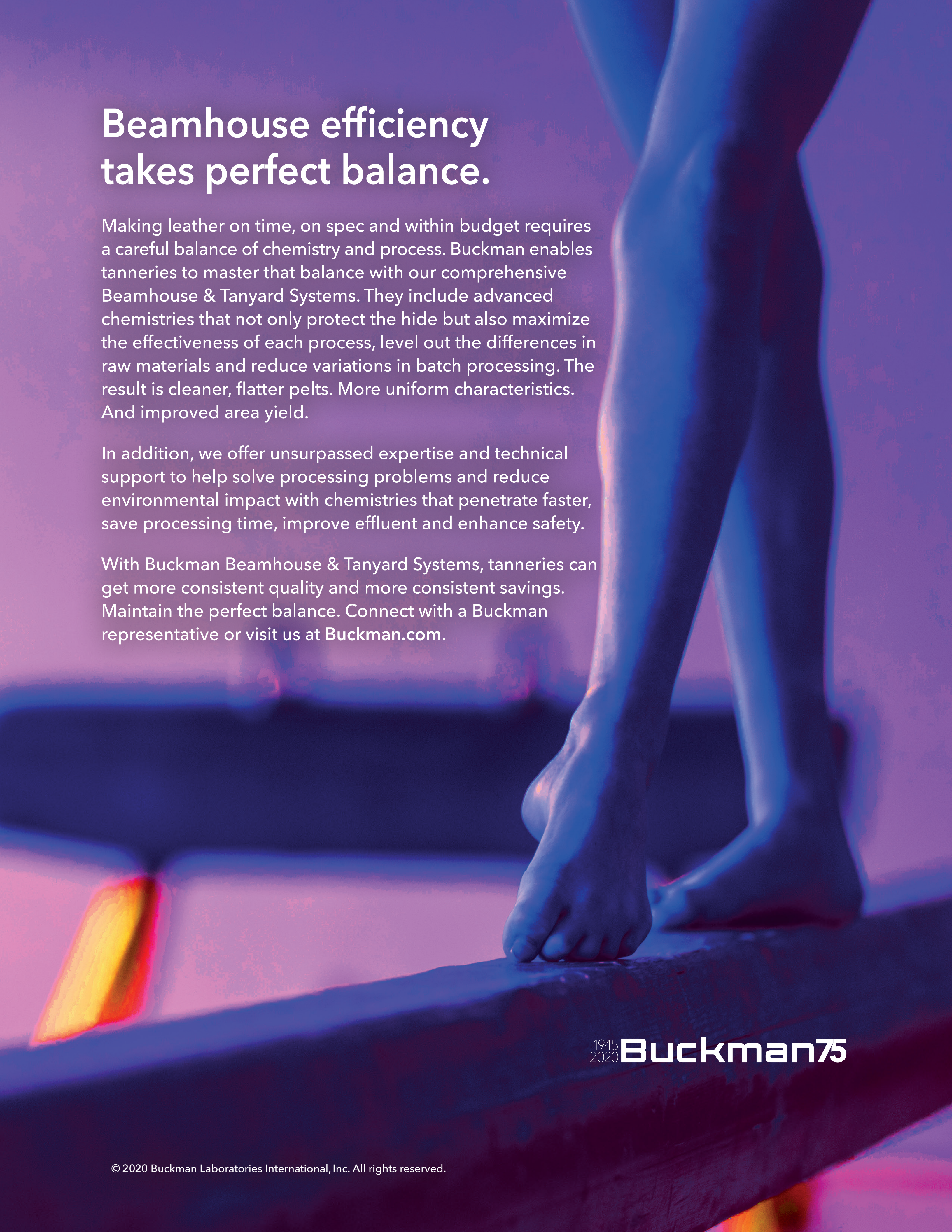

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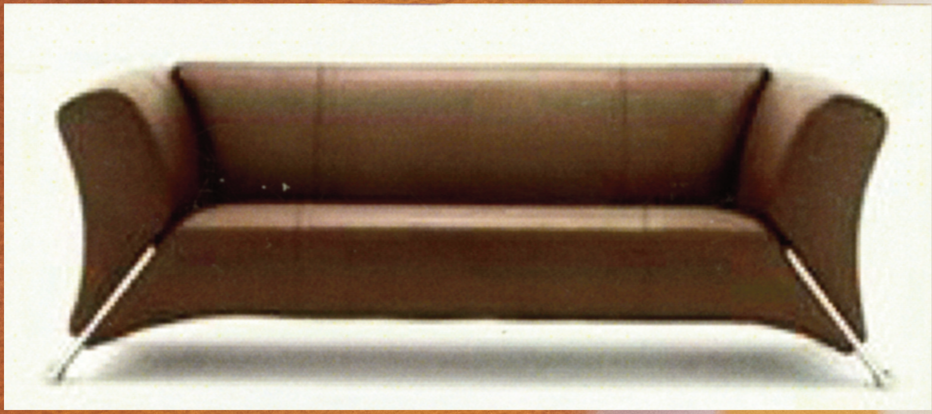

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Changes to the Collagen Structure using Vibrational Spectroscopy and Chemometrics: A Comparison between Chemical and Sulfide-Free Leather Process

by

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Abstract

Chemical and physical changes take place when hides and skins are processed to leather that affect the quality and strength of the material. Understanding the structure at each leather-making stage is the basis of this study but also intend to improve the process through a biochemical approach, employing a proteolytic enzyme for processing leather more cleanly with reduced environmental impact. Raman and ATR-FTIR spectroscopy in conjunction with chemometrics was used to investigate each leather-making stage from fresh green cattle hide to dry crust leather. The changes in proteins, lipids, nucleic acids and other biomolecules with leather processing was measured and reported using three novel Raman ratiometric markers, 920/1476, 1345/1259 and 1605/1476 cm^{-1}, to discriminate the structural changes in collagen of hide using standard chemical and enzymatic method. Amide I band was deconvoluted to investigate the collagen secondary structures using curve fitting by Gaussians function. The results of Principal Component Analysis are well-corroborated with the ratiometric markers of structural changes.

In summary, the results obtained demonstrates the potential of vibrational spectroscopy (Raman and ATR-FTIR), in conjunction with multivariate analysis, in generating universal biochemical signatures representing structural changes and helps in bringing the better ecofriendly method for greener leather process.

Introduction

A leather-making process is an approach to preserve skins and hides from decomposition and to provide flexible and robust leather. The process involves a series of mechanical and chemical treatments starting from the fresh skin to the final dry crust leather. With every chemical treatment, structural properties of the original skin are altered either by isolating the essential proteins, collagen, from the raw skins or hides or adding cross-linking agents in the later stages of processing to ensure stability and strength of the leather. It modifies the chemistry of collagen, which is widely known, but very little information is available about analyzing these processing effects on the collagen structure.

The skin acquired from the animal carcass after expulsion is termed as "fresh green". Skins are regularly salted to safeguard the skin prior to tanning briefly, and it caused some drying out of the skins. The next step of the leather making process is to rehydrate the skins by dousing and washing followed by a salt treatment using sodium sulfide termed as liming combined with suitable chemical or microbial enzymes termed as "bating", which separates and eliminates unwanted components. It assists with opening up the leather structure, allowing chemical penetration in ensuing stages. After treating with alkaline chemicals, the objective is to preserve the skin from putrefaction by adjusting to low pH and then treating with extreme acidic solution of sulfuric acid and sodium chloride. The skin obtained after this stage is termed as "pickled". The pickled skin can be preserved for a year and two**.** After pickling, a synthetic cross-linking agent is generally added, which helps with the following chrome tanning stage. The obtained pelts were then tanned using chromium sulfate and classified as "wet blue." The color and surface of the leather changes at this stage and will, in general, be excessively inflexible for most applications. Therefore, to make leather softer and fuller, vegetable or synthetic tannins, are added and the stage is termed as "retanned". Then the desired colors and fat liquors are added to provide the final finish to the leather and mentioned as "fat liquored." The leather obtained after is dried, softened, and is designated "dry crust" leather.[1,2]

The leather industry contributes to the economy significantly and poses a risk to the environmental due to release of various chemicals. Lime and sodium sulfide used at the liming stage of leather processing for unhairing skins and hides cause environmental pollution. The disulfide bonds in the keratin of hair and epidermis are broken down by sodium sulfide allowing the removal of hair from skin keeping the dermis intact and unaffected. The byproducts obtained from lime and sulfide produces foul smell and creates highly alkaline sludge which is hazardous to the environment[3] and poses a disposal problem for the leather industry.[4]

The role of enzymes in dehairing using alkaline proteases can serve as an alternative method to chemical unhairing to gain ecological advantages. Proteolytic enzymes are the most industrially utilized

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enzymes. Microbial proteases are more commonly used in the leather industry for being more environmentally friendly than chemical processes.[5-7] There are different classes of proteases used in leather processing, such as neutral proteases used in soaking, alkaline proteases for dehairing, and acid proteases for bating process.[8] There are several advantages of enzymatic unhairing which includes the removal of hair without affecting collagen content of dermis and no solubilizing of hair, decrease or even total disposal of sodium sulfide, great quality hair recuperation and improved working condition.[9]

In the present work, we have used vibrational spectroscopy techniques, Raman spectroscopy, and Attenuated Total Reflectance - Fourier Transform InfraRed (ATR-FTIR) to thoroughly characterize the samples obtained at each stage of leather making process. The aim was to analyze the variations in the collagen structure by comparing the samples obtained from standard leather-making protocol using hazardous chemical sodium sulfide and enzymes that are relevant for developing environmentally clean technologies. Both non-destructive techniques are fast, require minimal or no sample preparation, and are very specific and sensitive. Raman spectroscopy has the advantage of an extremely weak signal from water, so that the slight interference from water present in biological samples causes no damage to the sample and allows on-site detection with microscopes optics. If there is a decrease in the water signal, then the advantage of IR is its sensitivity to vibrations associated with amide bonds in proteins. The secondary structures of proteins influence the shape of the amide bands and IR spectroscopy provides extensive information regarding the structure of proteins. We have used an ATR-FTIR spectroscopy using a curve fitting method to analyze the secondary structures within the amide I band for collagen strength. The work is upheld with ratiometric and chemometric techniques, for example, Principal Component Analysis (PCA), which uses statistical and mathematical algorithms for quantitative data interpretation and allows for discrimination according to biochemical components in samples.

Experimental

Materials

New Zealand Leather and Shoe Research Association (LASRA) obtained raw cattle hide for processing in-house. Samples were obtained from the official sampling position (OSP) and removed from the same hide during several leather-making stages following the standard LASRA leather processing protocol as provided in the supplementary information (Table SI). These stages were termed fresh green, limed, pickled, wet blue, retanned, fat liquored, and dry crust. All samples had high moisture content, except dry crust, because they were obtained during the leather processing. The samples were sectioned using a freezing microtome (Leica CM1850 UV, Germany) to 60-µm thickness and transferred onto microscopes slides for Raman measurements. For FTIR measurements, samples were sliced into a thick piece and analyzed for FTIR scans.

Enzymatic depilation

A bacterium producing proteolytic enzyme was isolated and identified from tannery compost. Briefly, serial dilutions of the compost suspension were plated onto Luria broth agar plates with skimmed milk. After 24 hours, colonies surrounded by clearing zones were picked and cultured in LB broth at 25°C overnight. The identification of the bacteria was achieved by 16S rRNA gene sequencing, the results of which were analyzed using the Targeted Loci Nucleotide BLAST. From 16S rRNA gene sequencing and phylogenetic analysis, the proteolytic bacterium was identified to be *Vibrio metschnikovii*.

V. metschnikovii cryopreserved with glycerol at -80°C was activated by inoculation in 5 mL, 1:1000 ratio of LB broth and cultured overnight at 25°C on a shaking incubator. The activated culture was used to inoculate 3 L of freshly prepared fermentation media, which was then cultured for a further 48 hours. The crude enzyme extract was collected by centrifuging the resultant culture at 8000 g relative centrifugal force (rcf) for 20 min at 4°C. Ammonium sulphate precipitation was carried out to separate the total proteins from the crude protease extract, followed by centrifugation at 20,000 g for 30 min at 4°C.

The partially purified protease was rehydrated using 100 mL PBS and enzymatic depilation paint was prepared by mixing the enzyme solution with Solvitose (50 g/L) as a thickener. The resultant enzyme painting paste was applied on the flesh side of cowhide samples (20 × 20 cm^2), which were then kept at 25°C. Complete depilation was achieved after 24 hours of incubation. The depilated slats were processed into crust leather following standard LASRA protocol (Table SI), sectioned, and prepared for Raman and ATR-FTIR analysis.

Data acquisition and spectral processing

Raman spectra were acquired from samples under ambient conditions using a custom-built Raman microscope. 532 nm excitation laser (Laser Quantum Torus) was focused onto the sample using 0.65 NA and 40 magnification microscope objective. The laser power at the sample was around 10 mW. 532 nm Raman edge filter (12° incident angle) (Iridian Spectral Technologies, Canada) was focused onto a 50-micron entrance slit of a Teledyne-Princeton Instruments Isoplane 81 (FERGIE) spectrometer[17,26] and rejected the Rayleigh scattered light. Lightfield 6.1 software was used for the acquisition of spectra. Triplicate samples from each stage were prepared, and Raman spectra were acquired with an exposure time of 5 seconds per frame over 10 frames (each frame was saved separately) for data analysis. In total, 30 spectra were recorded for each leather-making stage.

ATR-FTIR spectra was recorded by attenuated total reflection (ATR) on a diamond crystal with 16 scans from each of the triplicate samples[17] using a Thermo Scientific™ iD5 Nicolet™ iS™5 Attenuated Total Reflectance - Fourier Transform InfraRed (ATR-FTIR) spectrometer.

The recorded spectra were preprocessed using the SciKit Learn package[13] algorithm written in Python 3.7. Baseline correction using asymmetric least-squares method was performed on average spectra using the python algorithm. Smoothing using the five-point Savitzky-Golay smoothing function to remove any spectral noise and normalization was carried out by dividing each point by the norm of the whole spectrum using Origin 2021b. Band areas of peaks of interest were determined by deconvoluting the peaks using curve-fitting function.[17,26] The most dominant changes observed in the amide I region was shown by deconvoluting the 1646 cm^{-1} (amide I) band. To account for the variability between the datasets of different leather stage samples, principal component analysis (PCA) was performed to extract meaningful comparison with significant variation of samples.

Results and Discussion

Lime serves to open-up the fiber bundles of hide matrix to the desired degree and helps to remove the hair completely. In the present method, alkaline proteolytic enzyme lime has been replaced to depilate the skin with good grain quality.[14] Hence, it is necessary to analyze the structural variations happening throughout the leather-making stages due to change in initial pre-tanning method and monitor the effect at the molecular level using Raman and ATR-FTIR spectroscopy.

Fundamental characteristic of Raman spectra of standard and enzymatic processed hide

The Raman spectra obtained from different leather-making stages are highly complex, consisting of a superposition of Raman scattering peaks from many biochemical constituents. Spectral variations were observed in the peaks highlighted (Figure 1). The bands were assigned based on already published literature and listed in detail in Table SII.[15-17] Peaks at 833-859,868 and 920 cm^{-1} are assigned to the collagen tyrosine, proline and hydroxyproline matrix, C-C skeletal of collagen backbone and the peak at 1002 - 1028 cm^{-1} is characteristic of phenylalanine. Other distinct peaks were observed at 1259 cm^{-1} (amide III ν(C-N) and δ(N-H) of proteins), 1345 cm^{-1} (CH_3CH_2 twisting of proteins and nucleic acids), 1445 cm^{-1} ($\delta(CH_2)$ deformation of proteins and lipids), 1553 (ν(C = C) of amide III), 1605cm^{-1} (δ(C = C) of phenylalanine) and 1655 cm^{-1} (amide I ν(C = O) of lipids). The Raman spectra of limed skin shows an overall increase in the intensity of all peaks with the maximum increase observed at 1095 cm^{-1} band and was about 3 times higher than for fresh skin. This band is attributed to lipids,[18] which might be released due to the breakdown of keratin and swelling of the skin producing sulfur compounds in conjunction with lime.

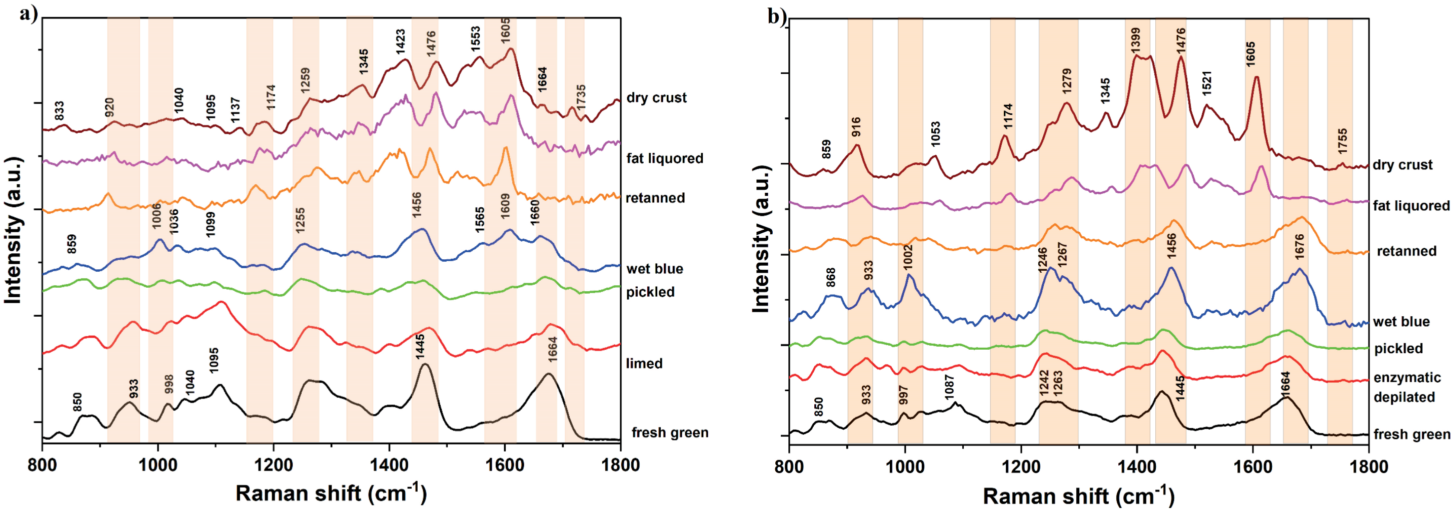

Figure 1. Raman spectra of different stages of leather-making using **a)** standard and **b)** enzymatic method.

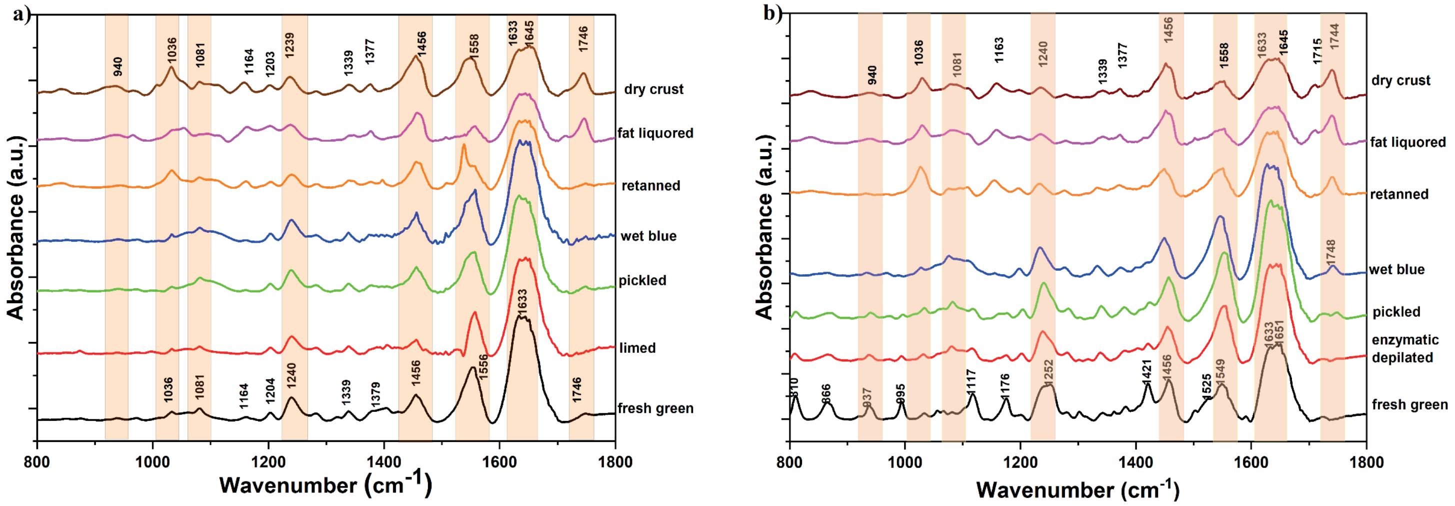

Figure 2. ATR-FTIR spectra of different stages of leather-making using **a)** standard and **b)** enzymatic method.

ATR-FTIR standard results also show two bands at 1036 and 1081 cm^{-1}, which can be attributed to sulfo groups due to the sodium sulfide[19] and lime used during liming. Raman spectra of enzymatic-depilated skin show a decrease in intensity than fresh skin. This could be due to an effective opening of the derma structure using the enzyme solution that degrades soft keratin, which is present in the roots of the hair, and amino acids during hide processing.

The band around 1745 cm^{-1} and the band at 1399 cm^{-1} can be attributed to ν(C=O) modes of lipids due to the addition of fatty acids in final stages of leather processing. There is a change in protein observed at every stage with a slight shift in Raman bands. Phenylalanine band at 1002 cm^{-1} shows a significant increase in intensity at the wet blue stage and almost disappears when it reaches the dry crust stage. This indicates that the addition of chemicals at every stage to remove non-collagenous protein, provide strength to leather, and alters skins biochemical structures. The Raman band at 1176 cm^{-1} appears at wet blue stage and becomes intense in later stages which might be due to interaction of chromium (Cr) with hide protein and addition of fatty acids forms complexes with carboxylate groups making the bond much stronger. This is quite evident at dry crust stage of enzymatic method with the onset of 1399 cm^{-1} band which attributes to C=O symmetric stretch and not observed in standard (liming) process. A change in the amide I position around 1664 cm^{-1} corresponds to collagen fibers explaining the force involved for reorganization of shape associated with the stretching and straightening of twists and turns. Such variations occur due to changes in the H-bonded network or other structural reorganizations in the collagen structure. The Raman spectra of each leather processing stage adds to change in protein vibration bands arising from polypeptide backbone (amide bands), intra- and intermolecular hydrogen bonds, and side chain groups. The disappearance of the amide I band in Raman spectra at final dry crust stage of standard and enzymatic method correlates with the presence of bulky side chains, such as, tyrosine and phenylalanine that would be relied upon to display spectra with a suppressed or absent amide I band.[20] It was assumed that the silence of amide I bands in Raman spectra results from the separating of peptide bonds from the metal (chromium)-protein surface after tanning stage. The closeness could depend on the length (bulkiness) of the amino acid side chains, which act as spacers between the fatty acid particles and the peptide bonds. The addition of fatty acids causes a change in the relative position of the domains, but complex formation does not change their conformational structure.

The characteristic bands obtained by ATR-FTIR results of the standard and enzymatic leather process method do not show any major shifts and can be visualized easily for slight changes. Raman band centered at 1664 cm^{-1} (Figure 2) represents the amide I of collagen, which usually consists of several secondary structures of amide I band (1600–1700 cm^{-1}), corresponds to C=O stretching vibrations, and the most sensitive part of the protein when determining the secondary structure.[21] The bond explains the backbone conformations and different type of secondary structure due to different C=O stretching. Therefore, ATR-FTIR spectroscopy is an effective tool for assessing the secondary structure of the protein. The amide I band is mainly used to quantify the secondary structure and conformational changes of proteins and polypeptides.[22] Fourier self-deconvolution (FSD) method, mathematical approach was used to isolate highly overlapping components of amide I, which originate from different secondary structural elements. While investigating the proteins, the amount of carboxyl groups reflects the ongoing situation. The IR results of fat liquored and dry crust (staked) showed that peak areas in enzymatic hide spectra at 1746 cm^{-1} emerged suddenly due to the increase in the amount of carboxyl groups with low hide pH of 3.6. The IR spectra of standard method showed characteristic absorption bands at 1748 cm^{-1} in pickled hide which disappear in chromated sample (wet blue) while two bands arising at 1717 and 1746 cm^{-1} at dry crust stage. The first disappearance and appearance of 1745 cm^{-1} band with shoulder band at 1717 cm^{-1} may be referred to the chromium complex while the band at 1745cm^{-1} may be referred to the free carboxylic groups of oligomers which might be created from fragmentation of peptide chains. Likewise, enzymatic method shows consistent appearance with increasing intensity of 1748 cm^{-1} band with fragmentation into two bands getting to the final stage. Each of the chemical treatments modifies the composition of collagen proteins and can be observed at peak 1664 cm^{-1}. With liming, deliming several non-fibrous proteins break down which can be seen in several secondary structures at pickled stage. It indicates the loss of the stability of collagen structure, which was regained after wet blue stage with addition of a few cross-links. After careful assessment, spectral variations were observed in the collagen region (1002-1680 cm^{-1}) indicating few shifts in peaks which could be due to intricacy of different biochemical components at each leather processing stage which create alterations in secondary structures leading to α helix, β sheet, cross links or random coils.[23,24]

Two spectral analysis techniques were employed to investigate the biochemical changes in the structural transformation from hide to leather. Few spectral differences were visible with naked eye but can be visualized using statistical techniques to identify a clear difference between the chemical and enzymatic processes.

- A univariate statistical method uses a single variable to investigates peak shifts, peak intensities, or peak area by calculating the ratios of peaks of interest for the interpretation of spectra.[25,26] This ratiometric analysis is a parameter for quantification and help the spectroscopists to understand spectral variations, and represent using statistical plots, such as bar plots in Figure 3. It can resolve variations due to sample morphology and other instrumental effects.[15]
- A multivariate statistical method - Principal Component Analysis, an unsupervised method that extracts the basic features from the full spectrum, based on the analysis of variance characteristics. Multivariate analysis does not make any priori assumptions about selecting the best variables for classification.[17,27]

Ratiometric analysis

Ratiometric analysis was performed using three novel Raman ratiometric markers, 920/1476, 1345/1259 and 1605/1476 cm^{-1}, to discriminate the structural changes in collagen of hide using standard chemical and enzymatic method. The final dry crust spectra were used for Raman and ATR-FTIR ratiometric analysis.

The amino acid contents (hydroxyproline and proline) are very important constituents in collagen because they play an important role in stabilizing the triple helical structure. From the ratio 920/1476 cm^{-1}, proline content (peak at 920 cm^{-1}) in collagen from enzymatic method was slightly higher than that in standard method, which suggests that collagen from enzymatic method, may have a more complex structure than that from standard method which determines higher collagen strength. Reduction of pH values at dry crust stage cause the decrease in intensity of the band located at 1476 cm^{-1}, which can be explained by H^{+} ion binding to nitrogen bond of side chain amino acids which helps in stabilizing the collagen structure. 1345 cm^{-1} is an indicator of protein alpha-helix orientation, the intensity of which is highly sensitive to molecular orientation. Increase in ratio of 1345/1259 cm^{-1} explains about structural changes in lipids and proteins due to interaction of chromium (Cr) with hide protein and addition of fatty acids makes it much stronger after interaction with carboxylate groups to form complexes. Such complexation enhances the quality of finished leather which is observed higher in enzymatic method. 1605 cm^{-1} contributed to phenylalanine (collagen proteins) and 1645 cm^{-1} to the amide I mode and may indicate a combined effect from changes of protein secondary structure.

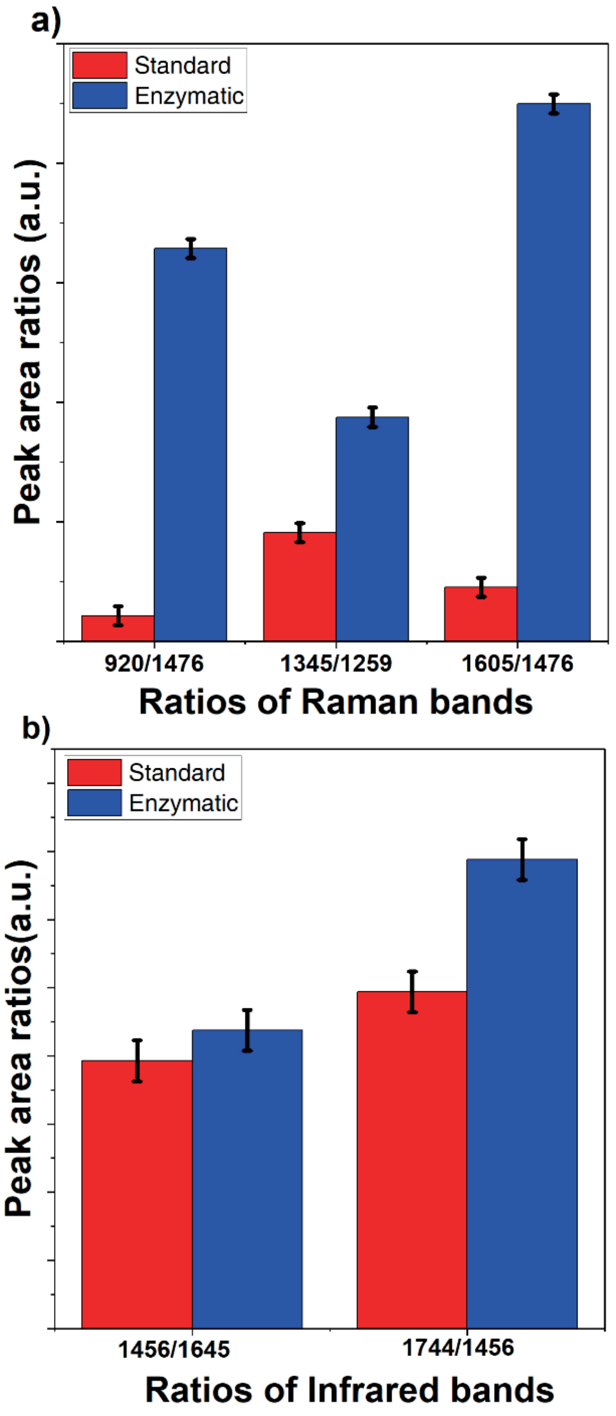

Figure 3. Peak area ratios of **a)** Raman and **b)** ATR-FTIR bands for standard and enzymatic method of leather making.

The other prominent FTIR marker used was 1744/1456 cm^{-1} (esters/CH$_2$ deformation of lipids). 1720–1750 cm^{-1} (νC=O) assigned to carbonyl or carboxyl compounds and considered as the evidence of hydrolysable tannins. It represents the absorption of the C= O bonds of the ester groups which shows an increase in lipid oxidation with the increase in 1744 cm^{-1} band after the addition of fat liquors. Standard method has higher lipid oxidation compared to enzymatic method. Such peaks highlight the changes in spectra for identifying carboxylic acids in fat liquors. These chemicals are responsible to cause chromium III (CrIII) to oxidize towards CrVI. These fragments can generate reactive oxygen species (ROS), resulting from lipid oxidation. The defined ratios indicate higher protein rearrangement and modifications using the enzymatic processing method, by opening-up of the fiber network without damaging the fibrous collagen to maintain collagen fibril strength. Therefore, Raman and FTIR ratiometric analysis supported the hypothesis that leather processing using enzymes can be considered as an effective and reliable method rather than using harsh chemicals toxic to human health and environment.

Alterations in collagen network

Raman spectra show disappearance of amide I band during the retanning state, which allowed investigating FTIR spectra for amide, I region sensitive to changes in the protein secondary structure. The amide I band of final dry crust leather has been picked out to compare standard and enzymatic method and are magnified in Figure 4.

The amide region of proteins is overlapped by many underlying bands (Figure 4). Deconvolution of hidden peaks using curve-fitting was carried out on FTIR data for components in the 1600 – 1700 cm^{-1} region to investigate the spectral changes in collagens secondary

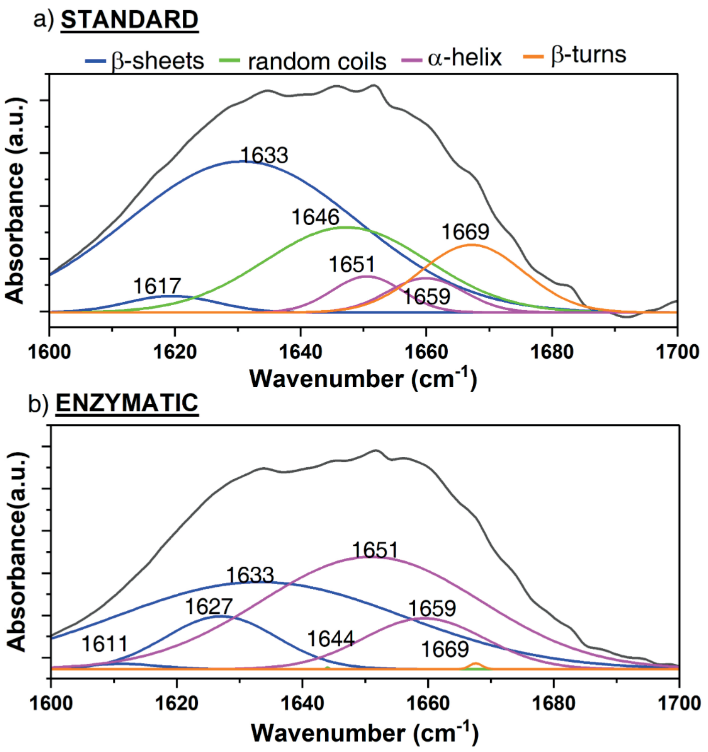

Figure 4 . Deconvoluted ATR-FTIR amide I region of standard and enzymatic method of leather making with the resolved underlying bands.

structures upon chemical treatment through different stages to final leather. Quantitative peak-fitting analysis of amide I band, as applied in this investigation, has been demonstrated helpful in studying the nature and the degree of protein conformation changes.[21,22]

Amide I band is the main contributor of protein in the skin.[28] Generally, proteins with α-helical content show an amide I band centered around 1650–1659 cm^{-1}, while those with predominantly β-sheets structures show the band at 1611-1635 cm^{-1}, a proportion of random coil or disordered structure attributes to proteins with an amide I band centered at 1642–1647 cm^{-1} and 1660~1700 cm^{-1} for β-turn.[29,30] The peak areas are calculated to identify the types of secondary structures in different samples, the component peaks, their assignment and percentage content of secondary structures is listed in Table I.

Due to variations in hydrogen bonding of polypeptide bond of α-helix, β-sheet, or disordered structures, there is a correlation between the FTIR band frequency and the proteins secondary structure. ATR-FTIR bands related to the peptide linkage (O−−C–N– H) are designated as the amide bands. The conformationally sensitive amide I band is contributed mainly by the C=O stretching mode of the collagen. It can form a hydrogen bond with the NH groups of another chains peptide bond (interchain) or of the same chain at different sequence positions (intra-chain).[31] If the hydrogen bonds are formed between the C=O and NH on the same chain, the polypeptide backbone is in α-helix, and the amide I band occurs at 1655 cm^{-1}. Deconvolution of amide I band spectral analysis reveals that α-helical content was highest in the enzymatic method whereas β-sheet structure was more significant in standard method. Significant shape changes in the amides complex peak area can be observed because of the peak change occurring at both the α-helical conformation and the β-sheet conformation. α-helix is the most intense component in providing the stability of collagen structure at the finished leather stage, which supports the enzymatic method as an effective treatment for improving the leather quality.

Multivariate analysis

Multivariate analysis, a statistical analytical technique, is used to find the trend and pattern in spectral data present in a large data set. Principal Components Analysis (PCA) is an unsupervised multivariate method used to reduce the dimensionality of large dataset into smaller dataset without any assumptions (Mehta et al. 2020). The grouped datasets of similar variability are ordered in accordance with highest importance as PC1(principal component 1), PC2(principal component 2), PC3(principal component 3) and so on. A score plot is used to demonstrate the discrimination among different datasets observed in the form of distinct clusters of samples.[32] For each unique variable, PCA creates coefficients that describe how much that variable adds to the basic functions.

PCA analysis was performed on the Raman and FTIR spectra obtained after analyzing the different leather processing stages. The PCA score plot of Raman spectra (Figure 5a, b) demonstrate the clustering of samples from each stage, highlighting specific variations in the structural profile of hide. This could be due to the opening of fibers at the liming stage and cross-linking during chrome tanning. There is a clear distinction between pre-tanning stages separated

Table I

ATR-FTIR Amide I band assignments for protein secondary structures

Amide I (cm^{-1})	Assignment	Average absorbance (%)	
		STANDARD	Enzymatic
1650 to 1659	α-helix	6	24
1611 to 1635	β-sheet	28	16
1669	β-turn	12	1
1642 to 1657	random coils	15	2

along PC2 and post tanning stages of retanning, fat-liquored and dry crust along PC1 are clustered together. PCA score plot of the enzymatic method shows not much discrimination during the pre-tanning process, but later stages of fat liquored and dry crust leather are very well separated along the PC1. Similar behavior was observed with FTIR results (Figure 6a, b), showing not much separation within samples obtained from the enzymatic method. This suggests that the enzymatic treatment is significantly dehairing the hide keeping the collagen intact without much alteration in the collagen network. PCA results well supports the findings obtained after ratiometric analysis, which represents that collagen content is high in the enzymatic method compared to the standard method. Also, well corroborated with the deconvolution of peaks analysis where the enzymatic method has higher α-helical content, which provides collagen strength. The standard method has a lime presence that opens-up the collagen fiber network to affect the penetration of further chemical treatments and provide a clear distinction between stages, whereas this was not observed in the enzymatic method. Figure 5c, d shows

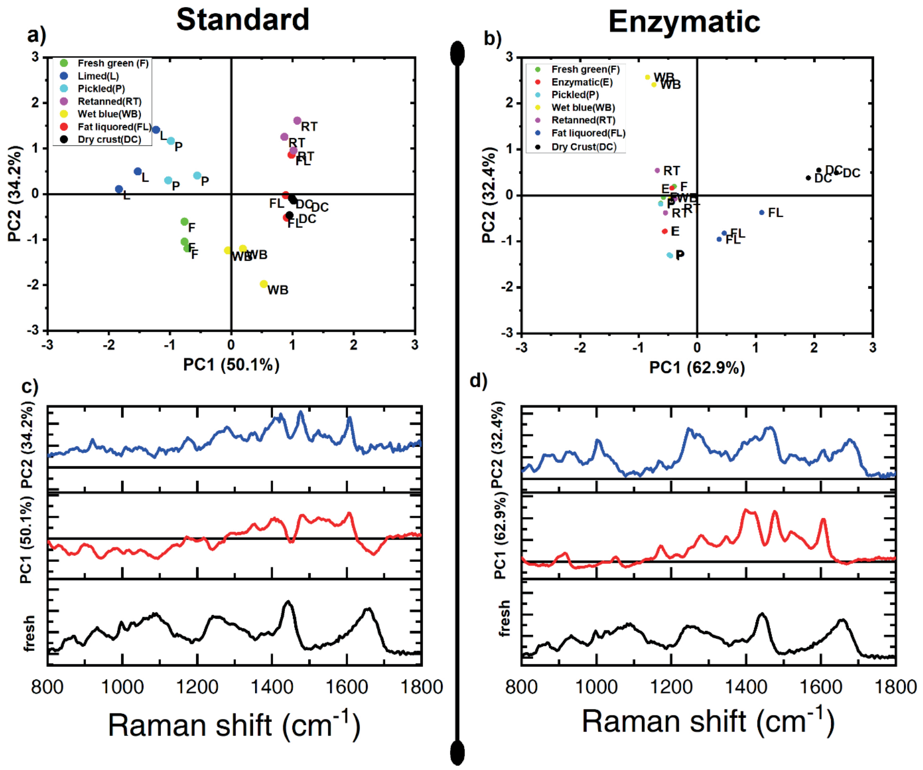

Figure 5. PCA score plots **(a, b)** and loadings plots **(c, d)** of Raman spectra of leather-making stages using standard and enzymatic method.

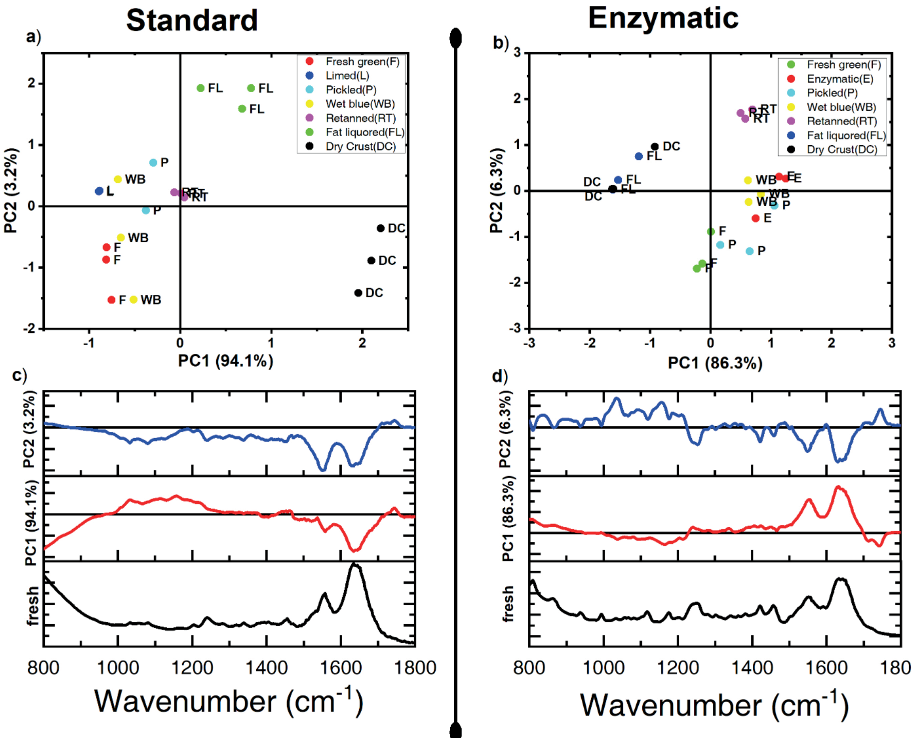

Figure 6. PCA score plots **(a, b)** and loadings plots **(c, d)** of ATR-FTIR spectra of leather-making stages using standard and enzymatic method.

the loading plots of the first two principal components of Raman spectra for standard and enzymatic methods. PC1 explains 50.1% of the standard and 62.9% for enzymatic data, while PC2 explains 34.2%, for standard and 32.4% for enzymatic method. The loading plots indicate which spectral bands contribute most to the variance described by the principal component. The fresh rawhide average spectrum is used as a reference for comparison of loadings. This gives an understanding of the origin of differences between the samples corresponding to spectral variations. The strong contribution in PC1 and PC2 is from the amide I stretch in the region of 1630 - 1690 cm^{-1}, mainly collagen proteins. Another contribution is from the 1456 cm^{-1} band, which is due to CH_3CH_2 deformation of proteins and lipids. PC2 also has spectral contributions from the amide I band, two significant peaks in the region of 1400-1500 cm-^{1}, which contributes to the deformation of proteins and lipids with C=N stretching from side-chain amino acids. Enzymatic method loading plot also has a similar contribution but with higher intensity with PC2 showing significant contribution around 1003 cm^{-1}, which is phenylalanine of collagen. ATR-FTIR loading plots (Figure 6c, d) for standard have a significant contribution from amide I band, but the enzymatic method has several minor contributions from collagen proteins in the lower wavenumber region.

Conclusion

The present work replaces the lime and sulfide with an alkaline proteolytic enzyme produced from bacteria *Vibrio metschnikovii* in leather processing. Raman and ATR-FTIR analysis was employed for comparing both chemical (lime and sulfide) and enzymatic methods. The results demonstrate that the extent of fiber opening using enzyme is better to conventionally lime and sulfide processed leathers by efficiently dehairing the process with retention of collagen protein. To the best of our knowledge, this is the first study done in depth using ratiometric and chemometric analysis to investigate the structural variations happening at every stage of leather process. Unsupervised multivariate analysis is well-corroborated with ratiometric results and provided an explicit discrimination in identifying subtle differences. Raman and ATR-FTIR, both vibrational spectroscopy techniques, can easily quantify the changes in biomolecules that impact leather quality, strength, and sustainability. This process also exhibits a significant reduction in pollution loads and eliminates the formation of H_2S gas and lime-bearing sludge, which are a significant concern for the environment. Therefore, these techniques can be used to monitor and evaluate any modification made to improve leather processing and paves the way to reduce environmental impact. In addition, results demonstrate better physicochemical properties of dried crusts with reduced pollution load, further confirms this enzymes potential for eco-friendly dehairing of animal skins in the leather industry.

Data Availability

The datasets generated and analysed during the current study are available from the corresponding author (Megha Mehta) on reasonable request.

Acknowledgements

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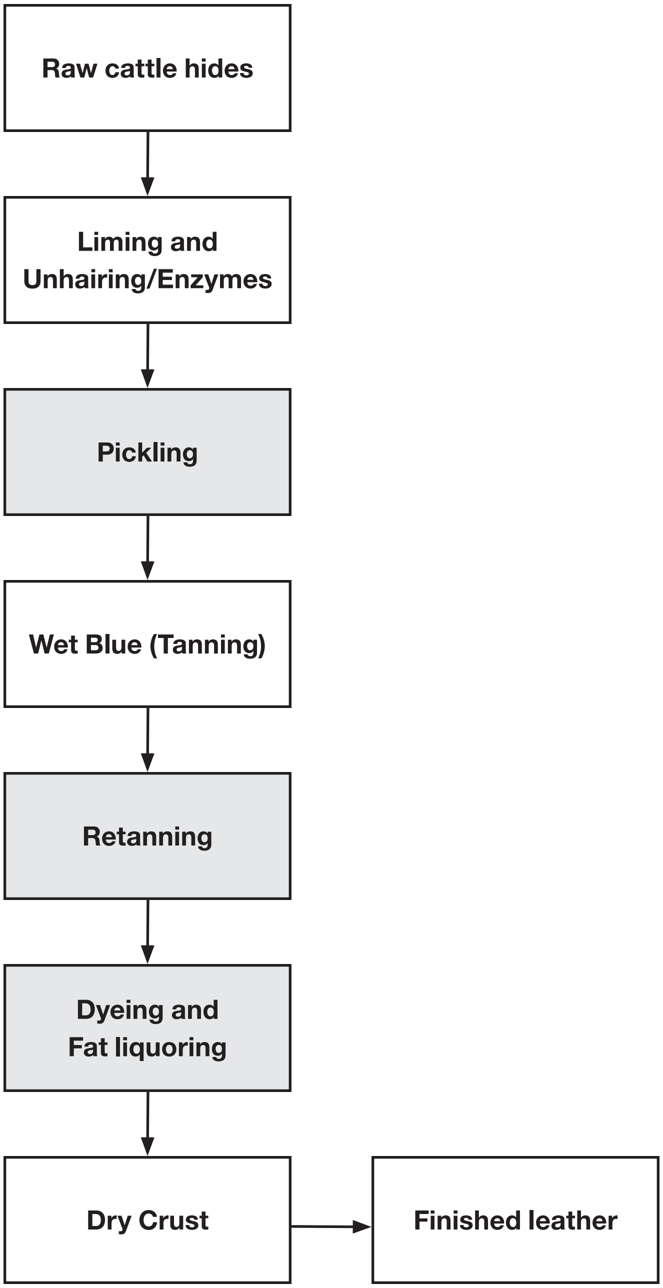

Figure S1. Main stages of leather processing

Table SI

Standard Leather Processing Protocol

Process	Amount (%)	Chemical	Temperature (°C)	Time (min)
Soaking	200	WATER	28	120
	0.5	Tetrapol LTN		
	0.05	TCMTB (30%)		overnight
Unhairing (Liming)	100	Water		
	1.5	Sodium hydrosulphide		
	2.5	Sodium sulphide		
	1.5	Lime		180 (overnight)
Washing	100	Water	28	
	100	Water		
	500	Water		
	0.5	Ammonium chloride		
Deliming	35	Water	30	
	0.5	Hydrogen peroxide		15
	2.5	Ammonium chloride		
	0.2	Tetrapol LTN		90
Pickled	0.04	Tanzyme		
	100	Water	35	20
	100	Water (Running wash)	20	20
	60	Water	20	
	8	Salt		10
	1.2	Sulphuric acide		
	10	Water	20	180
Tanning (Wet Blue)	6	Chrome powder		
	0.18	Fungicide		180 or O/N
Basification	0.3	MgO	40	O/N or until exhaustion
Neutralising	100	Water	35	60
	1	Tannigan Pak-N		
	1	Sodium formate		
	0.15	Sodium bicarbonate		overnight
Retanning	100	Water	35	10
	2	Tanicor		10
	3	Mimosa		15
	1	Dye		10
	50	Water		
Fat liquoring	1	Synthetic oil	50	
	1.5	Sulphirol		
	3.5	Polyol AK(Neatsfoot replacement)		45
Fixing	0.5	Formic acid (85%)	30	30
Dry Crust			15	

Table SII

Raman and ATR-FTIR observed bands for standard and enzymatic leather-making process.[15-17]

Peak position (cm^{-1})	Biochemical Assignments	Observable
833	Out of plane ring breathing tyrosine	Raman
859	Tyrosine/collagen	Raman
868	Proline	Raman
920-940	C–C stretching mode of proline and valine and protein backbone (α-helix conformation)	Raman & ATR-FTIR
1002 -1040	C-C phenylalanine of collagen	Raman & ATR-FTIR
1079-1099	S-O antisymmetric stretch; PO^{2-} stretch; C–C stretch of lipids,	Raman & ATR-FTIR
1053	C-O stretching, C-N stretching (protein)	Raman
1087-1099	Lipid, n(C-N), Phosphodioxy group in nucleic acids	Raman
1137	Fatty acids	Raman
1160-1179	C–H in-plane bending mode of tyrosine and phenylalanine (proteins); Cytosine and Guanine	Raman & ATR-FTIR
1203	Nucleic acids and phosphates	ATR-FTIR
1235-1300	Amide III of proteins (N-H bend in plane and C-N stretch) with significant mixing with CH_2 wagging vibration from the glycine backbone and proline sidechain	Raman & ATR-FTIR
1339-1345	CH_3 CH_2 side chain vibrations of collagen	Raman & ATR-FTIR
1370-1380	Lipids.	Raman & ATR-FTIR
1399	C=O symmetric stretch; fatty acids	Raman
1403-1457	CH_2/CH_3 deformation of proteins/lipids	Raman & ATR-FTIR
1476	C=N stretching	Raman
1521	-C=C- carotenoid	Raman
1532 -1575	Amide II (protein N–H bend in plane and C–N stretch) FTIR	Raman & ATR-FTIR
1600-1610	Phenylalanine, tyrosine, C=C (protein)Cytosine (NH2) protein assignment	Raman
1634-1695	Collagen Amide I (protein C=O stretch)	Raman & ATR-FTIR
1735-1755	CO–O–C ester carbonyl stretching vibration lipids	Raman & ATR-FTIR

Table SIII

Average Peak area ratios of Raman and ATR-FTIR spectra of standard and enzymatic method of leather processing.

Spectra	Average		
	Ratios	Standard	Enzymatic
RAMAN	920/1476	21.346	328.34
	1345/1259	90.65	187.34
	1605/1476	45.07	449.55
ATR-FTIR	1457/1645	39.266	43.74
	1744/1457	49.36	68.81

Evaluation of Antifungal Activity of Carbonate Solvent – Part:1

by
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Abstract

Antimicrobial agents have been used in leather manufacturing to prevent leather products from microbial contamination. In this work, the antifungal activity of green solvent such as propylene carbonate was investigated against the mixed culture of fungi isolated from wet-blue using broth dilution/well diffusion. A concentration of 5% and above (propylene carbonate) showed effective antifungal activity against the mixed culture of fungi and the efficiency of propylene carbonate on the mixed culture increased with increasing concentration/volume. Propylene carbonate exhibited fungistatic activity against the mixed culture of fungi but it lost its activity after a certain period and fungal growth was observed again. It was also found that 2% propylene carbonate in chrome tanning process effectively inhibited the fungal growth and the wet-blue can be preserved up to 30 days without any fungal attack.

Introduction

The use of fungicides in leather manufacturing is essential to prevent the damage caused by various fungal species on leather and leather products. Over the years there have been a number of changes in the fungicides used to treat leather against fungal attack. Phenyl mercury compounds like phenylmercuric acetate (PMA) were in common use and then chlorinated phenols like trichlorophenol (TCP), pentachlorophenol (PCP) and tetrachlorodibenzodioxin (TCDD) have emerged. By the early 1980s[1] the use of these compounds was restricted due to their high toxicity to humans and poor biodegradability. The fungicide 2-(thiocyanomethylthio) benzothiazole (TCMTB) became the new standard starting in the 1970s and remains in use today. In addition, other fungicides like orthophenylphenol (OPP), parachlorometacresol (PCMC), bromohydroxyacetophenone (BHAP), 4, 5,-dichloro-2-N-octyl-4-isothiazolin-3-one (DCOIT), 2-n-octyl-4-isothiazolin-3-one (OIT), 2-mercaptobenothiazole (2-MBT),[2] methylene bisthiocyanate[3] are also being used in leather making. Currently, these fungicides are slowly being replaced with carbamate (IPBC-iodopropargyl-N-butylcarbamate) and carbimate (CHED - S-Hexyl-S'-Chloromethyl-cyanodithiocarbimate) based molecules.[4] Due to the wide spectrum of activity and stability against the harsh chemical environment, CHED is finding a prominent place in the fungicide market. Besides, some attempts have been made to use environmentally and ecologically safer fungicides in leather making. Researchers studied the antifungal activity of *Anethum graveolens*/*Melaleuca alternifolia* essential oils against fungal species found on sheep lining leather[5] and also used *oregano oil* as antifungal agent in leather manufacturing.[6] Recently, an attempt has been made to develop a fungal resistant fatliquor from blended natural oils and used in leather manufacturing.[7]

Also, an attempt has been made to synthesize a novel hyperbranched polymeric antifungal agent from triazine molecules (cyanuric chloride and cyanuric acid) as an alternative to traditional fungicides.[8] Machuca et al., synthesized N-acetylated gemini surfactant and used it as an antifungal wetting agent in chrome tanning process.[9] Haibin et al., developed a new antimicrobial complex of Copper (II) with benzothiazole derivative and used in shoe lining manufacturing.[10] Recently, our research group developed a solvent selection tool and identified propylene carbonate as a potential candidate for alternative carrier medium to water.[11] Propylene carbonate (one of the cyclic carbonates) is classified as VOC exempt green solvent and also listed in the EPA (Environment Protection Agency) safer ingredients list.[12] It possesses a high flash point, low vapor pressure, high boiling point and also low toxicity (LD50: 21000 mg/kg of rat). Propylene carbonate (PC) is predominantly used as a carrier medium in ointment/cosmetic products,[13] nail polish remover,[14] dye carrier, electrolyte in Li-ion batteries[15] and CO_2/H_2S gas absorber. For the first time, propylene carbonate (PC) was successfully used as an alternative carrier medium to water in leather making and established a zero discharge chrome tanning process.[16] Further, it has also been found that there is no fungal growth on wet-blue processed in PC medium without adding any antifungal agent. In order to expand on previous research work, a systematic study has been carried out to establish the antifungal activity of propylene carbonate and its application in chrome tanning process.

Materials and Methods

Preparation of Mixed Stock Culture

Conventionally processed goat pickled pelt was converted into wet-blue without adding any fungicides in tanning. Subsequently, it has

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been allowed for 15 days for the growth of fungi at room temperature. After the fungal growth, the inoculum was taken by cotton swab and spread over petri plates containing solidified Potato Dextrose Agar Medium (PDA). The fungi grown stock culture (Mixed culture) was stored at -18°C.

Evaluation of Antifungal Activity by Broth Dilution Method

To verify the antifungal activity of propylene carbonate, a broth dilution test was conducted prior to the well diffusion method. The fungal inoculum was prepared from the stock culture with sterilized distilled water and vortexed. Subsequently, sterile potato dextrose broth (PDB) was prepared. Then, the mixed culture (50 µl) was added into the potato dextrose broth (Total volume - 5 ml) containing various percentages of PC viz., 0, 2.5, 5, 7.5 and 10%. Subsequently, the tubes were incubated in an incubator at 30°C.

Evaluation of Antifungal Activity by Well-Diffusion Method

Initially, the PDA medium was transferred to the petri plate and 100 µl of inoculum (mixed culture) was added into the PDA medium. Then, the mixture was uniformly mixed and allowed to solidify. On the plate, a well was made with the help of a 5 mm well cutter. Subsequently, different volumes of PC viz., 0, 25, 50, 75 and 100 µl was separately added into the each well.

The plates were incubated at 30°C for 3 days. A circular zone of inhibition is observed as a result of antifungal activity of PC and the diameter of the zone is noted.

Mode of Inhibition

The mixed culture was added into the broth containing 7.5 & 10% PC and incubated at 30°C for 3 days. Then, 100 µl of the inoculated sample was added into the broth medium (No PC added) and observed for fungal growth. On the other hand, the plating was done with the same procedure as followed in well diffusion method. Then 75 and 100 µl of PC was added into the well and incubated at 30°C. After zone formation, the plate was left undisturbed for further incubation up to 5 days to examine the recurrence of fungal growth and to determine the mode of action of PC.

Evaluation of Antifungal Efficiency on Wet-Blue

To determine the effectiveness of propylene carbonate as an antifungal agent in chrome tanning process, wet-salted goatskin was conventionally pickled and chrome tanned. After chrome tanning, the liquor was completely drained and the wet-blue was divided into 6 parts and subsequently marked. Then different percentages of PC viz., 0, 1, 2, 3, 4 and 5% w/w along with 10% water was added and agitated for 30 min. A small piece was taken from each percentage and placed in a sterilized petri plate. All the plates were kept at room temperature (30°C) for observation of notable fungal growth.

Result and Discussion

Preparation of Stock Culture

The fungal spores were taken from two different places of wet-blue and subsequently, plating was done. After 72 hrs, a dense fungal growth was observed (Figure 1). It is evident from Figure 1 that the fungal growth pattern and its morphology of the spores collected from two different places are similar.

Evaluation of Antifungal Activity by Broth Dilution Method

Preliminarily, broth dilution test was carried out to evaluate the antifungal activity of propylene carbonate. The mixed culture treated with different percentages of propylene carbonate and its growth after 72 hrs is shown in Figure 2. It is clear from Figure 2 that the fungal growth is being observed in test tubes containing 0 and 2.5% PC. However, the fungal growth in 2.5% PC treated solution is relatively low when compared to 0% PC. No fungal growth was observed in the remaining test tubes where the concentration of PC was above 2.5%.

These results suggest that the PC shows effective antifungal activity against mixed culture isolated from we-blue and its efficiency increased with increasing concentration of PC.

Evaluation of Antifungal Activity by Well-Diffusion Method

The well diffusion method was carried out and the size of zone formation was taken as a marker to analyze the antifungal activity

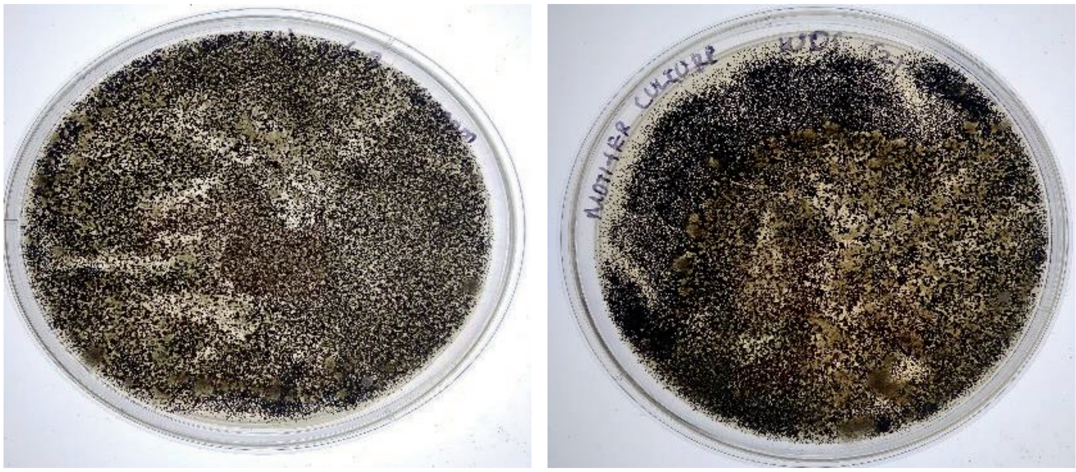

Figure 1. **(a)** and **(b)** Culture taken from two different position of chrome tanned leather

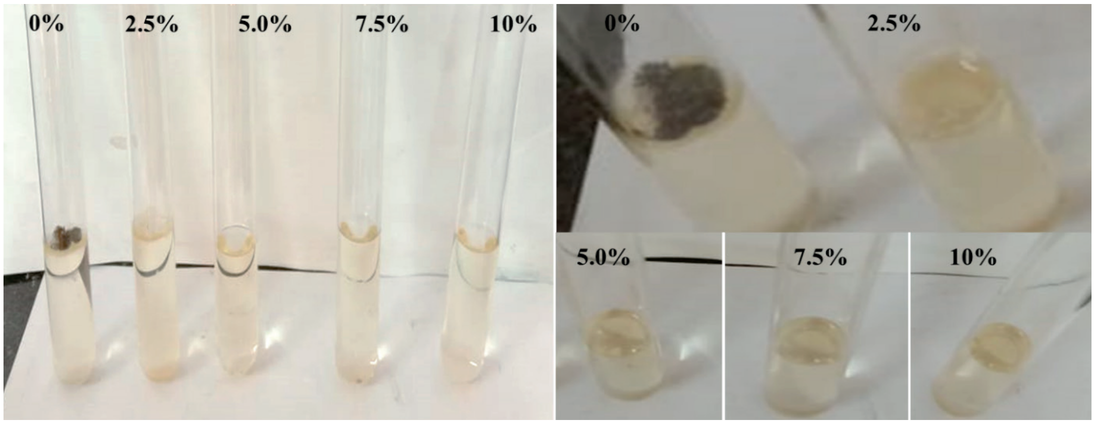

Figure 2. Mixed culture treated with different concentrations of propylene carbonate

of propylene carbonate. It is evident from Figure 3 that the zone formation was observed after 72 hrs from plating. However, the size of zone formation is varied depending upon the volume of propylene carbonate added into the well. It is also evident from Figure 3 that no zone formation was observed for 0% PC (Positive control) and no fungal growth was observed for negative control (no PC and no mixed culture). The size of zone formation for different volumes of propylene carbonate is shown in Figure 4. It is evident from Figure 4 that the zone of inhibition linearly increased with increasing the volume of propylene carbonate added into the well. The size of zone formation for 25 µl is 14 mm and it increased to 20 mm for 100 µl.

Mode of Inhibition

Generally, the antimicrobial agents used in leather manufacturing cause permanent damage to the microorganism. The phenolic based antimicrobial agents reversibly adsorbed on the cytoplasmic membrane of microorganisms which initially inhibits the transport of nutrients and eventually deteriorates the cell wall leading to microbial death.[17] On the other hand, the heterocyclic antimicrobial agent like TCMTB is an electrophilic substance that chemically reacts with the nucleophilic substance of cellular materials and causes cell death. Therefore, antimicrobial agents exhibiting static action would be safer. In order to study the mode of inhibition, the mixed culture was incubated in the presence of 7.5 and 10% PC. No fungal growth was observed up to 72 hrs. Then, incubated culture was sub-cultured (100 µl of an incubated sample taken) in the absence of PC and fungal growth was observed after 72 hrs. Further, two plates (7.5 µl and 10 µl) were incubated for 7 days and observed for recurrence at zone inhibition area. It is evident from Figure 5 that the fungal growth was observed in the zone of inhibition region. From the results, it may be concluded that the antifungal activity of propylene carbonate is fungistatic

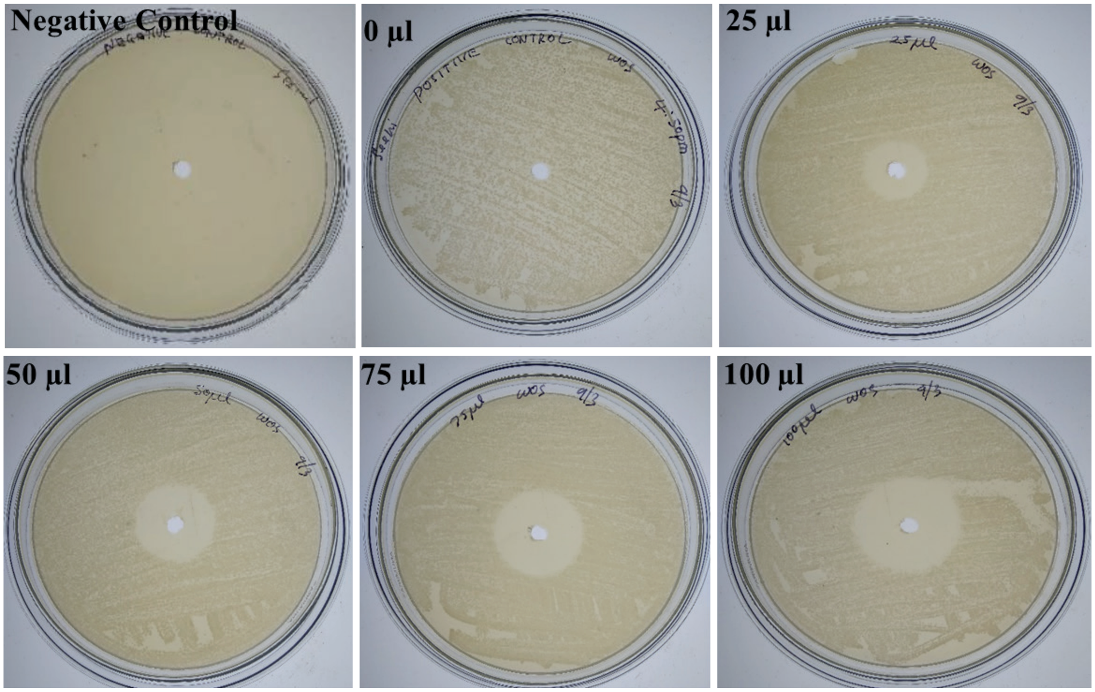

Figure 3. Antifungal activity of propylene carbonate – well diffusion method

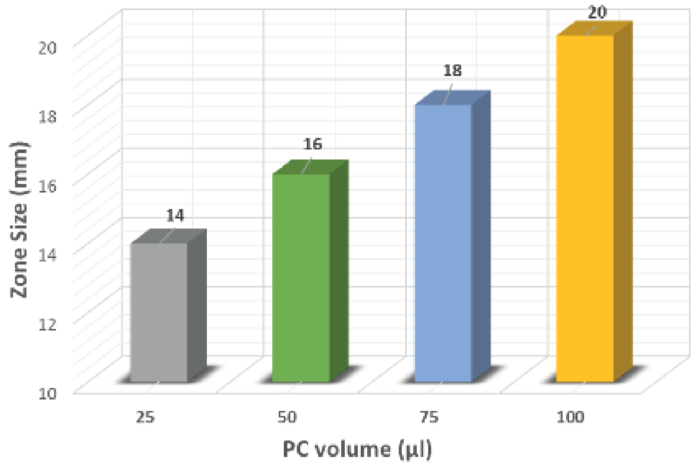

Figure 4. Zone of inhibition for different concentrations of PC

and it only arrests the fungal growth for a particular period of time without killing them.

Evaluation of Antifungal Efficiency on Wet-Blue

In order to study the antifungal efficiency of different percentages of propylene carbonate viz., 0, 1, 2, 3, 4 and 5% w/w were employed in chrome tanning. The wet-blue treated with each percentage was monitored for a period of 30 days and shown in Figure 6. On day 5 fungi formation was observed for 0% PC and no fungal growth for the remaining percentages. However, on day 14 growth was observed for 1% treated wet blue. No fungal growth was observed for remaining concentrations up to 30 days. Thus, it is clear from this observation that fungal growth shows resistance to lower concentrations of PC (1%) and the growth is inhibited with high concentration of PC (2% and above).

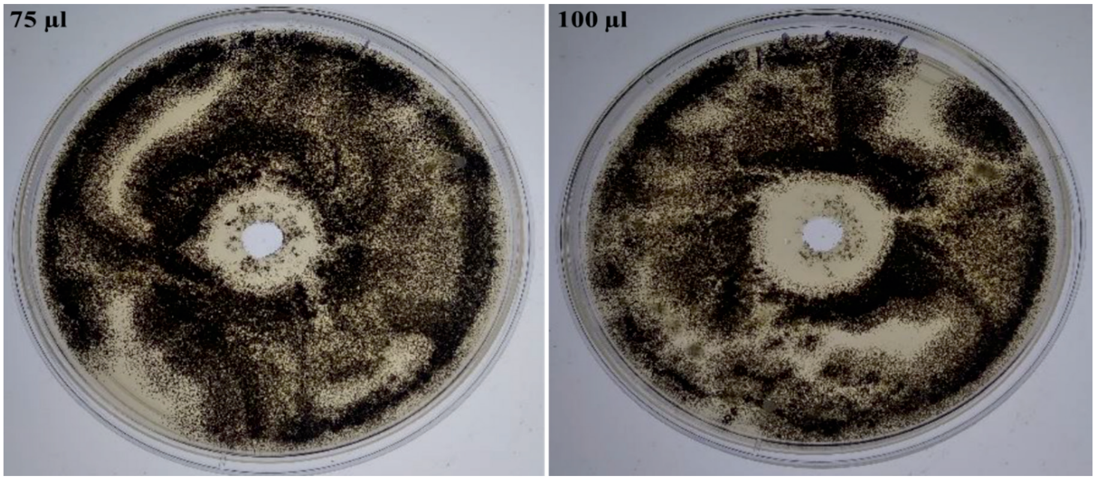

Figure 5. Mode of inhibition of propylene carbonate — Recurrence Test

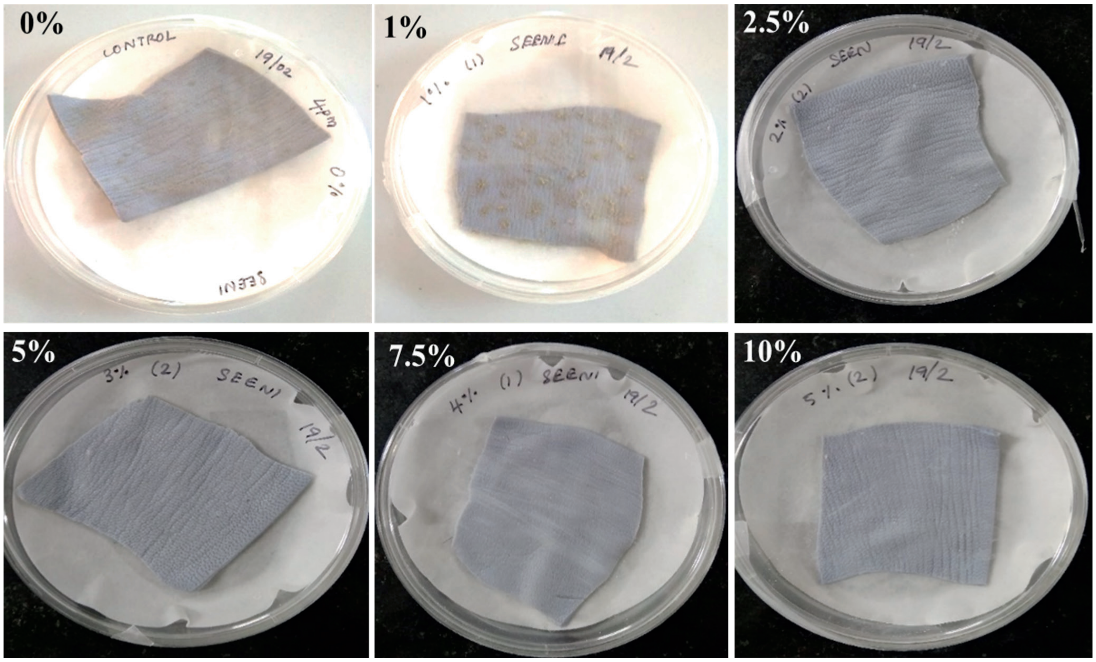

Figure 6. Wet-blue treated with different concentration of PC (After 30 days).

Conclusions

In this work, the antifungal activity of propylene carbonate against a mixed culture isolated from wet-blue was successfully evaluated. The results from the broth dilution method and well diffusion method suggest that the efficiency of propylene carbonate increased with increasing concentration/volume. It was also found that the mode of inhibition of propylene carbonate is fungistatic action and recurrence occurred after a certain period. The effective concentration of propylene carbonate in chrome tanning process is 2% and wet-blue may be preserved 30 days without fungal growth. Therefore, the propylene carbonate (green solvent) would be an ideal alternative for the conventionally used harmful antimicrobial agents.

Acknowledgement

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Biosorption of Chromium from Spent Semi-Chrome Liquor: Part 1 Effective Pollution Abatement using *Bacillus cerus*

by

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Abstract

The current study focuses on the isolation of *Bacillus cerus* from mangrove rhizosphere and its ability to treat semi-chrome process liquor of upper leathers. This strain has been identified by its molecular characteristics (16s rRNA sequencing) and confirmation has been obtained from neighbor joining tree. Minimum inhibitory concentration of the strain has been found to be 50 ppm. The growth pattern of this organism has been investigated in the presence of chromium, which showed the bacterial strain can grow luxuriantly at 50 and 100 ppm concentration of chromium. Biosorption study has been conducted at different concentrations (50, 100, 150, 200 and 250 ppm) of chromium. The biosorption capability of *Bacillus cerus* has been found to be 80.78, 73.19, 65.86, 59.44 and 39.27% for 50, 100, 150, 200 and 250 ppm respectively. Chromium sorption from the semi-chrome process liquor by *Bacillus cerus* has also been investigated, which showed a reduction of 76.15, 68.56, 61.63, 56.29 and 36.51% against 50, 100, 150, 200 and 250 ppm of chromium. Sorption characterization has been carried out by FTIR (Fourier Transform Infra-Red spectroscopy) and SEM (Scanning Electron Microscopy) analyses and the results confirmed the presence of sorption of chromium in *Bacillus cerus*.

Introduction

The leather industry has been considered as one of the most promising export industries worldwide for the past four decades. Vegetable tanning and chrome tanning are the major conventional tanning methods. However, chromium and vegetable tannins discharged from the tannery have become crucial despite several developments that have been studied in the tanning industry. The leather sector has developed several technologies for the uptake of chromium during tanning from 40-50% to near 100% in chrome tanning process.[1] However, the semi-chrome process wastewater poses a challenge in separating chromium from vegetable tannins by conventional methods. The footwear industry continues to follow the vegetable tanning processes with the traditional semi chrome processes due to the need for specific properties in these leathers, pastel shades and light weight leathers.[2] The vegetable tanned leathers are treated with basic chromium sulphate in a conventional manner during the semi-chrome tanning process. Vegetable tannins and higher molecular weight polyphenols, sugars etc. are present in the spent effluent in addition to chromium.[3] The conventional precipitative recovery of chromium involves formation of stable complexes with metal ions (o-dihydroxyphenyl chelating functional groups in the tannin molecules to form stable complexes with metal ions and settle in the spent liquor.[4] The acid used for treatment and reuse of chromium in effluent treatment of semi-chrome process generates quinonoid and other degraded organic compounds, which cause stains in the leathers when reused in tanning.[2] The introduction of metal ions like zinc and barium in the form of zinc tannate and barium tannate result when chemical methods are used.[2,3] Precipitation methods with heavy metal ions, polyvinylpyrrolidone, adsorption onto zirconium pillared clay, gelatine, collagen as well as electrochemical treatments have been reported for the removal of vegetable tannins.[4-9] Hence, there is a need to develop a low cost biological material which can potentially remediate the chromium from the semi-chrome process liquor.

Biosorption is a technique where the living organism encompasses itself in the accumulation of heavy metals from the wastewater.[10] Bacteria is one of the promising candidates to adsorb the heavy metals. Bacterial cell wall has the peptidoglycan layer, which has potential binding sites to accumulate heavy metals.[11] Hence, the present study focused on *Bacillus cerus,* a marine bacteria isolated from mangrove rhizosphere, to bring about selective biosorption of chromium from vegetable tanning containing liquor.

Experimental

Collection of effluent

The spent semi-chrome liquor after basification from the semi-chrome tanning process has been collected from a leading commercial tannery manufacturing upper leathers in Chennai, India. The process of tanning employed for making upper leathers has been wattle for vegetable tanning followed by chrome tanning after stripping process. The spent semi-chrome liquors have been analysed for chromium content, vegetable tanning content and chemical oxygen demand (COD).[12,13]

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Isolation and identification of chromium resistant bacteria

Soil samples have been collected from mangrove rhizosphere and serially diluted and spread over nutrient agar plates. Morphologically different organisms have been selected and sub-cultured. Based on the chromium tolerance in the presence of vegetable tannins, the relevant strain has been selected for further studies. Biochemical characteristics of the strain has been evaluated by standard methodology.[14] Genomic DNA extraction has been carried out using the standard method.[15] PCR amplification has been carried out using universal primer and sequenced, and the obtained sequences have been submitted to NCBI and an accession number has been obtained.

Minimum inhibitory concentration

Minimum inhibitory concentration of chromium against the selected organism has been evaluated using agar well diffusion method. The bacterial culture has been grown in the nutrient broth and have been spread on the nutrient agar. Wells have been prepared on the nutrient agar and filled with chromium solution at different concentrations. The cultured plates have been incubated at 30°C for 24 h.

Bacterial growth evaluation

Growth pattern of the selected bacteria in the presence of chromium has been evaluated, 1% of the bacterial strain has been introduced into the nutrient broth containing micro-titer plate with different concentrations of chromium liquor as well as spent semi-chrome liquor (50, 100, 150, 200 and 250ppm). Plates have been incubated at 30°C and bacterial growth has been monitored at regular intervals at 600 nm.

Biosorption analysis

Chromium sorption capability of *Bacillus cerus* has been studied following the method of Saranya et al.[16] with minor modification. Nutrient broth has been prepared with chromium and spent semi-chrome liquor by introducing 1% *Bacillus cerus* culture into the broth. All the samples have been incubated in shaking condition (150 rpm) for 24 h at 30°C. The culture broth has been centrifuged and the incubated pellets have been separated and supernatant has been digested with nitric acid and perchloric acid. The digested samples have been analyzed using ICP-OES. Bacterial biomass has been introduced to the diluted semi-chrome liquor that has been collected from the commercial tannery along with nutrient broth and incubated at 30°C for 24 h. The biomass has been separated from the media for all the samples and used for further analysis.

Characterization

Biosorption ability of *Bacillus cerus* has been characterized by FTIR and SEM. The bacteria strain from the broth has been collected from centrifugation and dried at 60°C for 18 h. Dried powders have been used for characterization. Functional groups involved in the sorption study have been assessed using FTIR spectrometer (JASCO 4200) and morphological changes have been observed under microscopical analysis (PHENOM ProX). Bacterial culture without chromium has been maintained as control.

Results and Discussion

The present study emphasizes the assessment of the chromium sorption capability of the newly isolated chromium resistant

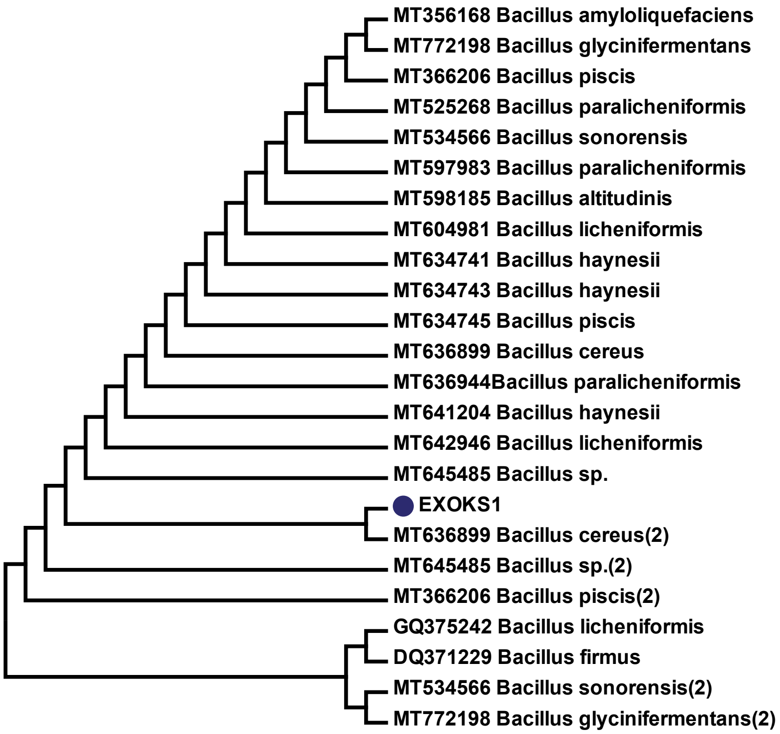

Figure 1. Phylogenetic tree of the strain *Bacillus cerus*

Table I

Characteristics of spent semi-chrome liquor

Parameter	Value
Spent semi-chrome liquor	
pH	3.5±0.1
COD (ppm)	7056±42
Total chromium as Cr (ppm)	2140±18
Vegetable Tannins (ppm)	4280±16

bacteria *Bacillus cerus*. This strain was isolated from a marine source mangrove rhizosphere and identified using biochemical and molecular characteristics. Sequence of the strain has been submitted to the NCBI and an accession number has been obtained (MW463427). Phylogenetic tree has been drawn (Figure 1) using MEGA 6.0 software to confirm the identification. *Bacillus cerus* has been identified as a gram positive, aerobic, spore forming rod shaped bacteria[17] as the potential candidate for the removal of chromium in the presence of vegetable tannins. The characteristics of spent semi-chrome liquor from the commercial tannery is given in Table I. The COD, vegetable tannin content and chrome content of the semi-chrome liquor taken for studies are 7056, 4280 and 2140 ppm, respectively. The spent semi-chrome liquor has been suitably diluted and used for the study.

In earlier days, researchers focused on the isolation of bacteria from industrial water or from the leather dump yard due to their ability to create resistance against certain pollutants for their survival.[18] In the present study, as a novel approach, we isolated the bacterial strain from the mangrove rhizosphere, which has the possibility to adsorb only chromium from the semi-chrome liquor containing vegetable tannins. Marine bacteria rather than terrestrial bacteria have been considered as efficient community due to their tolerance capability. The natural existing bacteria from marine source of mangrove rhizosphere have also been proved that they also have equal potential to resist developed organisms.

To identify chromium resistance of the strain, minimum inhibitory concentration has been investigated against different concentrations of chromium (50, 100, 150, 200 and 250 ppm). The zone of inhibitions were measured as 0.1, 0.3, 0.4, 0.5 and 0.6 cm against 50, 100, 150, 200 and 250 ppm of chromium respectively. Followed by the minimum inhibitory concentration test, growth pattern of the *Bacillus cerus* has been evaluated in the presence of same concentrations of chromium, which has been used for the minimum inhibitory concentration evaluation. Growth pattern of the bacterial strain showed increasing concentration of chromium promote inhibition of bacterial growth (Figure 2). Maximum growth has been noted at lower concentration (50 ppm) of chromium for up to 48 h and maximum inhibition has been observed at 250 ppm. Bacterial growth has been found to be higher at 24h at 100, 150 and 200 ppm concentrations, which can be attributed to contact time and binding site availability of the bacteria. A similar growth pattern has been observed in the presence of semi-chrome liquor also (Figure 3), but the bacterial growth has been comparatively lower than the above study, where only chromium resides. This can be due to the lower availability of bacterial species for the vegetable tannins present in the semi-chrome liquor at the same concentration and duration of chromium uptake.

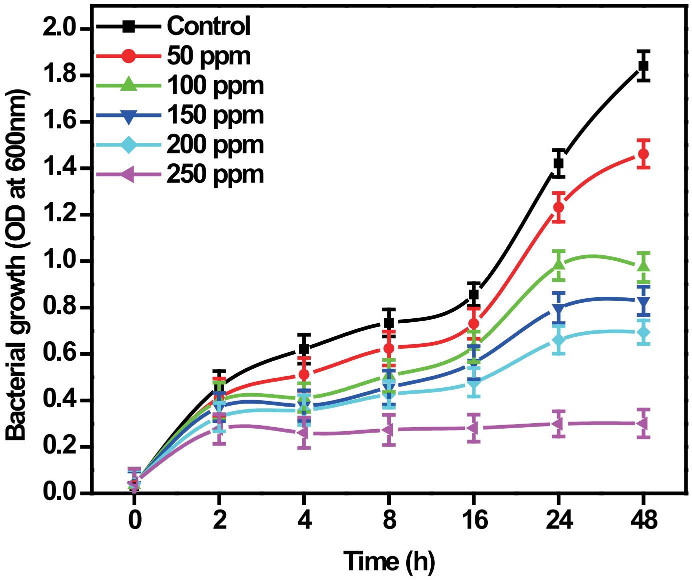

Figure 2. Growth analysis of the *Bacillus cerus* with and without presence of chromium

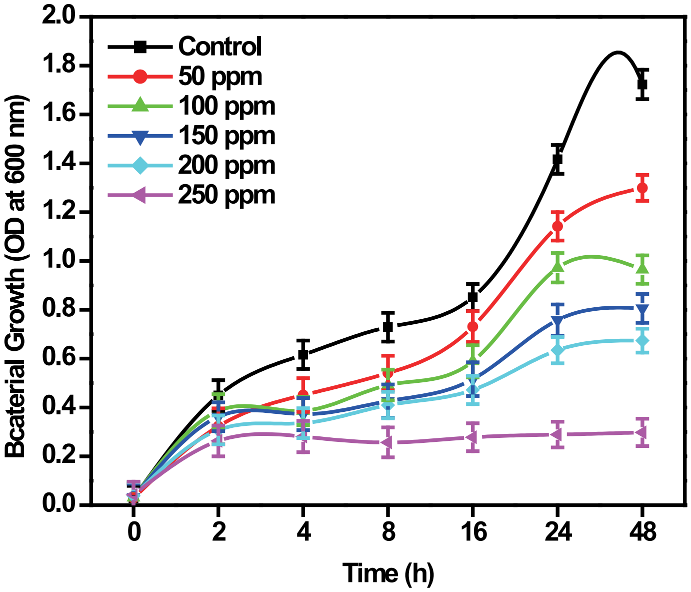

Figure 3. Growth analysis of the *Bacillus cerus* with and without presence of semi-chrome liquor at different concentrations of chromium

The removal of Chromium by the bacterial strain has been centered on the variation in concentration of chromium. The observed chromium removal percentage has been 80.78, 73.19, 65.86, 59.44 and 39.27% for 50, 100, 150, 200 and 250 ppm concentrations of the chromium respectively (Figure 4). Chromium removal percentage of the strain *Bacillus cerus* has been gradually increased from high to lower concentrations that could be due to availability of more reactive sites, which could have fastened up the sorption process. This can be supported by the previous study, which indicated that increasing metal concentration can increase the biosorption capability of bacteria up to certain threshold level.[19] Removal of chromium from semi-chrome liquor has also been evaluated at the concentration of 50, 100, 150, 200 and 250 ppm. Biosorption percentage calculated on post treatment of semi-chrome liquor, resulted in 76.15, 68.56, 61.63, 56.29 and 36.51% of chromium absorption at respective concentrations of 50, 100, 150, 200 and 250ppm (Figure 5). Maximum biosorbed biomass was taken for further characterization (50ppm).

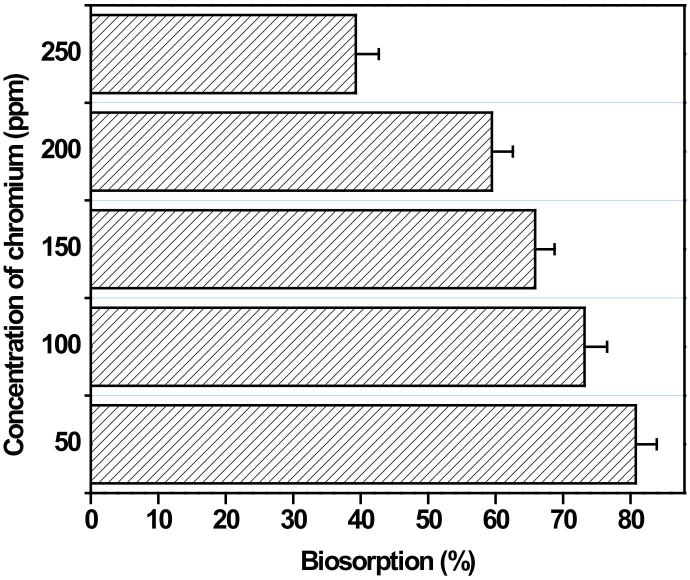

Figure 4. Biosorption capability of *Bacillus cerus* at different concentration of chromium

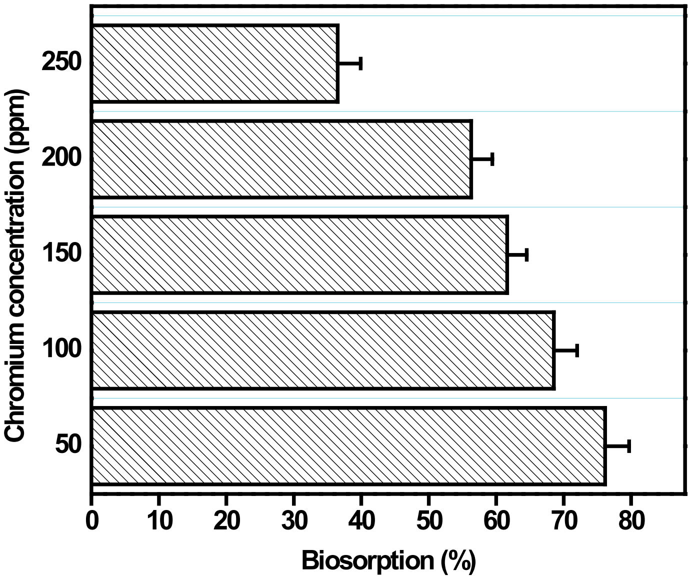

Figure 5. Biosorption capability of *Bacillus cerus* from semi-chrome liquor

The probable mechanism of biosorption is that the bacterial cell walls consist of various charged functional groups which act as binding sites for heavy metals. Hence, in order to identify the functional groups which play a role in the sorption process, FTIR analysis has been carried out. The FTIR spectrum analysis has been investigated for *Bacillus cerus* with and without chromium in the range of 400-4000cm^{-1} to determine the possible functional groups involved in the sorption process (Figure 6). The functional groups responsible for biosorption and the shift in spectrum have been analyzed and given in Table II. The change in shift of peak is at 3647, 1839 and 13396 from 3282, 1640 and 1366 cm^{-1} respectively, which are responsible for –OH, C-O/C-C and –CO/-OH stretching bands. One more peak has been observed at 801 cm^{-1} in metal absorbed biomass, which arise due to metal ligand vibration and the presence chromium. Vibrational spectrum between control and experimental samples concludes the presence of sorption on the bacterial biomass.

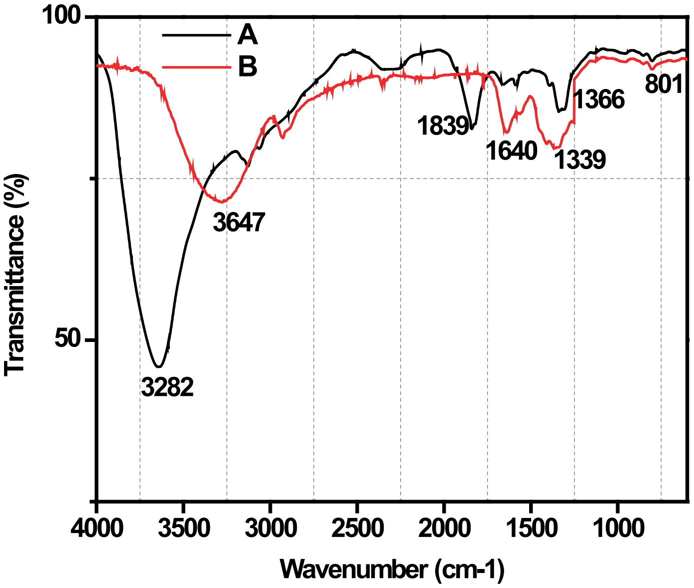

Figure 6. FTIR of the bacterial strain **(A)** Control **(B)** After Experiment

Table II

IR peaks of responsible functional groups involved in biosorption

S.No.	With chromium	Without chromium	Corresponding Functional Group
1.	3647	3282	-OH stretching
2.	1839	1640	C-O/C-C stretching
3.	1339	1366	-CO/-OH Stretching
4.	–	801	Metal ligand vibration

The results of the present study are also supported by some earlier investigations with respect to metal uptake by bacteria.[20,21] The morphological changes of the bacterial strain due to the sorption has been analyzed using scanning electron microscopy. *Bacillus cerus* exposure to the chromium in the presence of vegetable tannins resulted in morphological changes as seen in Figure 7. Due to the production of extracellular compounds by the bacterial strain, there are distinguished changes in communal structure, showing that the cell wall has been wrapped with heavy metals.

Conclusion

Chromium removal from the spent semi-chrome effluent is a challenging issue in wastewater management. The present study concludes that the newly isolated culture *Bacillus cerus* from mangrove rhizosphere (a marine bacteria) showed the minimum inhibitory concentration at 50 ppm and the growth analysis study determined that the strain could grow rapidly for up to 200 ppm concentration. The strain efficiently removed chromium up to 80.78% at 50 ppm concentration of chromium. Biosorption capacity of *Bacillus cerus* has been observed at 76% from the semi-chrome liquor at a concentration of 50 ppm and has been supported by the characterization evidence of FTIR and SEM results. The study concludes that the strain *Bacillus cerus* can be a potential candidate for the treatment of chromium from vegetable tannin containing semi-chrome process effluent.

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Author Contributions

All the authors contributed to frame the manuscript. SK: Experimental performance and writing original manuscript; KB: Writing review; SVK: Technical review, editing, supervision.

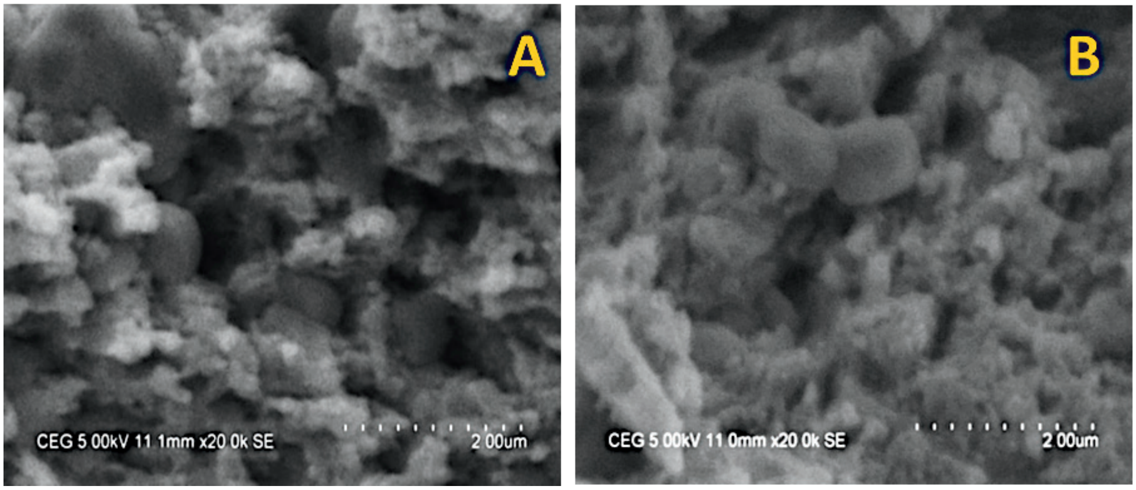

Figure 7. SEM micrograph of *Bacillus cerus* **(A)** Control bacteria **(B)** Experimental bacteria

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Typical Defects of Natural Phospholipid Fatliquors in Leather Industry and Their Solutions

by

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Abstract

Leather made with soybean phospholipid fatliquors is prone to problems such as yellowing, elevated hexavalent chromium content, and undesirable odor. In this study, the aforementioned typical defects of soybean phospholipid fatliquors were investigated in respect to the main components, the antioxidants and the unsaturation degree of the natural soybean phospholipid. The results showed that the oxidation of soybean phospholipid is the primary source for its yellowing, elevated hexavalent chromium content, and undesirable odor. The volatile aldehydes produced by lipid oxidative rancidity are the main components of the undesirable odor. The purification of natural soybean phospholipid through removing the non-phospholipid components cannot solve the problems caused by oxidation of phospholipid. Furthermore, as a typical natural antioxidant existing in natural soybean phospholipid, tocopherols can restrain the oxidation of phospholipid to a certain degree, however, the dissolving out and destruction of tocopherols at high temperature in the phospholipid purification process can lead to more obviously oxidation of phospholipids. Additionally, the oxidation defects of phospholipid cannot be completely resolved by adding extra tocopherols, even at high dosages. The research finds that the defects of soybean phospholipid fatliquors can be thoroughly solved by increasing the saturation degree of lipid through addition reaction, the suggested iodine value of phospholipid products is lower than 20 g I_2/100 g.

Introduction

Phospholipids are phosphorus-containing fats isolated from plant or animal, such as oilseeds, grain germ, egg yolks and fish. The main source of phospholipids in plants is soybeans,[1] which is large in yield, rich in phospholipid content, and available at larger scale due to lower cost compared to synthetic phospholipids.[2] Today, "Phospholipid" has become the trade name for the mixture of phosphatides typically used in a vast range of foods, feed, pharmaceutical and technical applications.[3-4]

Fatliquoring is an important unit in leather making processes; it can endow leather with good softness by lubricating its fibers.[5] Phospholipids are surface active amphiphilic molecules, which comprise a polar head group and a lipophilic tail that can be used as the primary basis for a fatliquor. The compound fatliquor with soybean phospholipids as primary basis can give leather excellent softness, fullness, wetting properties and so on. Moreover, soybean phospholipids have low production cost and good biodegradability.[6] To this end, soybean phospholipid is often used as a raw material in the production of soft leather, such as sofa, garment and gloves. However, there are still facing some problems during the application of phospholipids in leather making industry. For example, soybean phospholipid fatliquored leather is prone to yellowing, elevated hexavalent chromium content, and conferring offensive odors after a period of time use and storage. However, the primary causes for these problems are still lack of systematic research until now, which restricted the further development of phospholipid fatliquor applications.

Concentrated soybean phospholipid is a by-product of soybean oil refining industry, which is mainly composed of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidyl acid (PA) and phosphatidylinositol (PI), as well as glycolipids, triglycerides, free fatty acids (FFAs) and sterols.[7-8] Additionally, it also contains some amounts of protein, tocopherol, carbohydrates, waxes, pigments, and so on.[9-10] The phospholipid components in concentrated soybean phospholipids can be extracted by organic solvents, of which acetone is the most commonly used. Acetone insoluble substance (AI) is generally considered as phospholipid component, and acetone soluble substance (AS) is non-phospholipid component, which is mainly including pigments, carbohydrates, free fatty acids, soybean oil and tocopherol.[11]

Phospholipids provide nutritional value owing to the high poly-unsaturated fatty acid composition, including oleic, linoleic, linolenic and arachidonic fatty acids.[12] A high degree of unsaturation can cause phospholipids to be prone to oxidative rancidity,[13] which affects the application effect of phospholipid fatliquor and may be the main reason for the defects of phospholipid fatliquor. It is important to note that other components, such as the acetone insoluble substances, pigments, glycolipids, FFAs and antioxidants, may also lead to the abovementioned phenomenon.

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FFAs are products of hydrolysis and degradation of triglycerides in concentrated soybean phospholipids, and their concentration is considered an important quality indicator of oil products. FFAs can reduce the surface tension of soybean oil, increase the diffusion rate of oxygen into the oil, thereby increasing the oxidation rate of oil.[14] In oil-water emulsions, FFAs can concentrate on the surface of the emulsion droplets and attract transition metals that promote the oxidation of oil. In addition, some experimental studies have shown that FFAs can promote the oxidation of oil as well.[15-17]

Tocopherol (VE) is a typical natural antioxidant to improve the oxidative stability of oil[18] and protect other oxidized substances from being destroyed. VE can be added to the oils as an antioxidant, which not only prevents the oxidation reaction, but also improves the nutritional value, and reduces the risk of cancer, cardiovascular disease and inflammation in human body.[19-20] In recent years, with the safety of synthetic antioxidants being generally questioned, vitamin E, a natural fat-soluble antioxidant, has attracted the edible oil industry's attention.[21-22]

The elevated hexavalent chromium content and undesirable odor of phospholipid fatliquored leathers are serious problems. The qualitative and quantitative detection of leather volatile compounds is very important to study the causes of the above problems. Headspace Solid phase Micro extraction (HS-SPEM) technology is a new technology for collecting volatile substances.[23-24] HS-SPME consumes less sample volume than that of Solid phase extraction (SPE). Additionally, HS-SPME integrates extraction, enrichment and sampling, and it can minimize the loss of analyte components to ensure the accuracy of the testing results.[25] Therefore, the main volatile substances in the fatliquored leather might be able to be determined and analyzed by headspace solid phase microextraction-gas chromatography-mass spectrometry (HS-SPEM-GC-MS) method.

In order to solve the typical defects of soybean phospholipids fatliquors, the effects of the main components (AS and AI), FFAs, tocopherols and oil saturation degree on the odor, hexavalent chromium content and yellowing resistance ability of phospholipid fatliquored leather were investigated in this study. Methods for solving these defects were explored, which can provide some direction for the development of high-quality phospholipid fatliquors.

Materials and Methods

Main instruments

Pump heat circulation stainless steel drum GLSD-40 (Wuxi Ronghao Leather Machinery Manufacturing Co., Ltd.), SHZ-B constant temperature water bath oscillator (INASE Scientific Instrument Co., Ltd.), Color i5 desktop colorimeter (X-rite Co., Ltd), GX-503-A yellowing resistance tester (High-speed Railway Testing Instrument Co., Ltd.), GT-303 Softness Tester (Gotech Testing Machines Co., Ltd.), Agilent 1260 High Performance Liquid Chromatograph (Agilent Technology Co., Ltd.), Trace Q9000 GC-MS (ThermoFisher Scientific Co., Ltd.).

Experimental materials

(α) tocopherol (HPLC) was purchased from Aladdin Biochemical Technology Co., Ltd.; UB60 Concentrated soybean phospholipid and soybean oil are industrial grade and provided by Guangzhou Hisoya Biological Science & Technology Co., Ltd.; Cattle wet blue leather was provided by Zhejiang Tongtianxing Group Joint-Stock Co., Ltd. All the other chemicals used for the analysis were of analytical grade and other chemicals used for leather processing were of commercial grade.

Purification of concentrated soybean phospholipid

50 g of concentrated soybean phospholipids (UB60) and 100 mL of acetone were added to a 250 mL round-bottom flask and stirred at 50°C for 30 minutes. Samples were placed in an ice-water bath for 5 minutes to reduce the solubility of phospholipids in acetone, then, suction filtered. Sediments were placed in a vacuum drying oven at 40°C to volatilize the acetone until constant weight. Repeat the above acetone washing operation to obtain phospholipids with different washing times.

AS was obtained by distilling the collected filtrate to remove acetone. Filtrates with different times of acetone washing were mixed. Acetone contained in the mixture was removed by distillation at 80°C and rotary evaporation at 40°C, respectively. The products were labeled as AS-80 and AS-40.

Preparation of modified phospholipids

15 g of concentrated soybean phospholipid was added to a 250 mL round-bottom flask, 15 mL of dichloromethane (or petroleum ether) was added. Samples were placed in a constant temperature water bath with an electronic stirrer, 150 r/min. Then, a certain amount of bromine was slowly added and reacted for 2 h. After the reaction, the solvent was recovered by distillation to obtain the finished product.

Determination of AI and AS content and phospholipid characteristic indexes

Content determination of AI and AS

2 ± 0.001 g of phospholipids and 30 mL of acetone were added to a beaker and stirred with a glass rod in a water bath at 50°C for 2 minutes. Samples were placed in an ice-water bath for 5 minutes to reduce the solubility of phospholipids in acetone at low temperature, then, suction filtered. The above operations were repeated 5 times. The obtained phospholipid and filtrate were dried in vacuum at 40°C to constant weight.[26] The contents of AS and AI were calculated according to their respective proportions (*W*/*W*) in phospholipids.

Determination of acid value

0.2~0.5 g of samples (concentrated phospholipids, AI or AS components) were added to a 300 mL Erlenmeyer flask. 70 mL of ether, 30 mL of neutral ethanol and 0.5 mL of phenolphthalein indicator solution were added and mixed. 0.1 moL/L potassium hydroxide-neutral ethanol standard solution was used to titrate the sample until the solution turned pale red and did not fade within 30 seconds as the endpoint. The acid value is obtained by dividing the mass of potassium hydroxide consumed by the mass of sample *(mg KOH/g)*.

Determination of iodine value

The iodine values of the samples were determined according to the method in the literature.[27]

Determination of (α)tocopherol content by HPLC-DAD

(α) tocopherol content in samples was determined through HPLC-DAD method. Chromatographic conditions: Agilent C18 column (250 mm × 4.6 mm, 5 μm); Diode Array Detector (DAD) detector (wavelength was 294 nm); Column temperature was 30°C and the mobile phase was pure methanol. The flow rate was 1 mL/min and the injection volume was 10 μL.

Fatliquor compounding and fatliquoring process

Fatliquor compounding

Phospholipids, mineral oils, surfactants and water were weighed and mixed according to the mass ratio of 5:1:10:6. the pH value of emulsion (fatliquor is compounded with water in the mass ratio of 1:9) was adjusted to 7-8 by using sodium hydroxide solution (4.0 mol/L).

Fatliquoring process

Cattle wet blue with the thickness of 1.8 mm-2.0 mm was selected and sampled symmetrically along the backbone. Samples were wetted, retanned and neutralized to pH 6.0 ± 0.1 as per the standard leather making process. Samples were fatliquored with different fatliquors at 50°C with 100% of water and 20% of fatliquoring agents for 2 h. Then, the pH was adjusted to 3.6 ~ 3.8 by using formic acid solution (1:20). The crust leathers were naturally dried. The odor, color difference value and hexavalent chromium content were determined.

Physical and chemical properties of the crust leather

Organoleptic investigation of odor

According to the "Geely Automobile leather odor determination method", the specific determination steps are as follows: 100 mm × 200 mm of crust leather samples were put into a sealed bag and placed at room temperature for a certain time after sealing. The mouth of the bag was slightly opened and the assessor's nose is 2-3 cm away from the mouth of the bag to evaluate it.

An evaluation team composed of 4 members conducted a fatliquored leather odor sensory assessment. Using a 9-point system (1=no peculiar smell, 5=moderate peculiar smell, 9=strong peculiar smell) the average was used to arrive at a quantitative assessment for each sample.

Thermal aging of leather and hexavalent chromium determination

100 mm × 200 mm of crust leather samples were placed in an oven at 80°C for 12 h, then cooled at room temperature for 30 min. The contents of hexavalent chromium in the crust leathers were determined according to the reported method.[28]

Color difference values determination

(a). Illumination treatment: 50 mm × 50 mm of crust leather samples were placed in an GX-503-A yellowing resistance tester at 50°C, and irradiated with a 300W power bulb for 24 hours. The color difference values of leather samples before and after illumination were detected by a colorimeter.

(b). Dry heat treatment: 100 mm × 200 mm of crust leather samples were placed in an oven at 120°C for 24 hours, and the color difference values of the leather samples before and after heating were detected by a colorimeter.

HS-SPEM-GC-MS detection of volatile gases

The volatile compounds in leathers were collected and studied by HS-SPEM-GC-MS. The volatile compound mass spectrum data was automatically vetted through the atlas library and the components with low content and low confidence were discarded. Finally, the relative percentage of volatile compounds was determined by gas chromatography peak area percentage method.

2 g of sheared leather samples (approximately 2 mm × 2 mm) were put into a 20 mL headspace bottle that sealed with a rubber stopper of PTFE film and equilibrated at 80°C for 1 h. After equilibration, the extraction needle was used for headspace adsorption for 30 minutes and directly inserted into the GC-MS injection port for desorption at 250°C for 5 min.

Chromatographic conditions: capillary column (30 m × 0.25 mm × 0.25 μm); inlet temperature was 250°C; carrier gas was helium (99.999%), and the flow rate was 1.2 mL/min. Heating program: the initial temperature was 80°C; then, heated up to 230°C at 5 °C/min; Injection mode: Split less injection. Mass spectrum conditions: electron bombardment ion source (EI); electron impact energy (70 EV); Ion source temperature was 230°C. Interface temperature was 250°C and the full mass scan range was m/z 50-500.

Results and Discussion

Main components and characteristic indexes of soybean phospholipids

Concentrated soybean phospholipid is extracted from soybean meal, and its components are complex. According to its solubility in acetone, it can be divided into acetone insoluble substance (AI) and acetone soluble substance (AS). Generally, AI is considered as the main phospholipid component, and AS is non-phospholipid component. In order to explore the effects of these two components on the properties of phospholipid fatliquor, concentrated soybean phospholipid (UB60) was washed with acetone for several times to obtain PL1 phospholipid (1 time washing), PL2 phospholipid (2 times washing), PL3 phospholipid

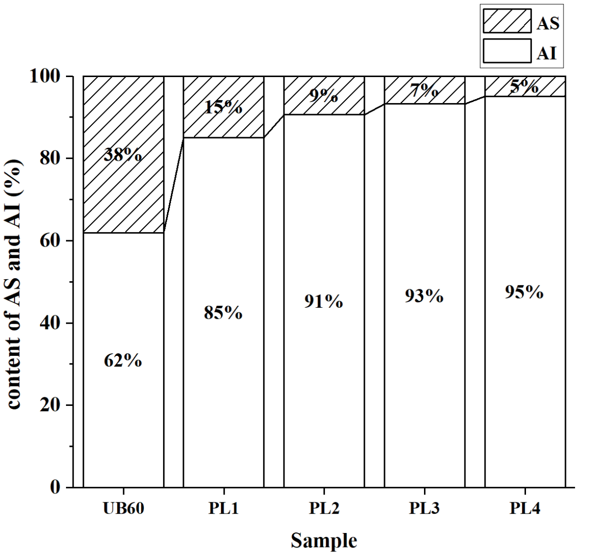

Figure 1. Changes of Contents of AI and AS in Phospholipids

(3 times washing), PL4 phospholipid (4 times washing) and acetone soluble substances (AS) respectively. The AI and AS content, acid value and iodine value of phospholipids were tested.

Content and acid value of AS and AI

With the increase of acetone washing times, the color of phospholipids gradually became lighter and the fluidity was lost. After 4 times of washing, PL4 phospholipids obtained was a yellow powder. The changes of AS and AI contents in phospholipids with washing times are shown in Figure 1.

Figure 1 shows that during acetone washing, components in concentrated soybean phospholipid, such as soybean oil and pigments, were dissolved in acetone, and concentrated soybean phospholipids were purified. The content of AI in phospholipids were increased with the increasing of washing times. The AI content of PL4 phospholipids reached up to 95% after 4 times of washing.

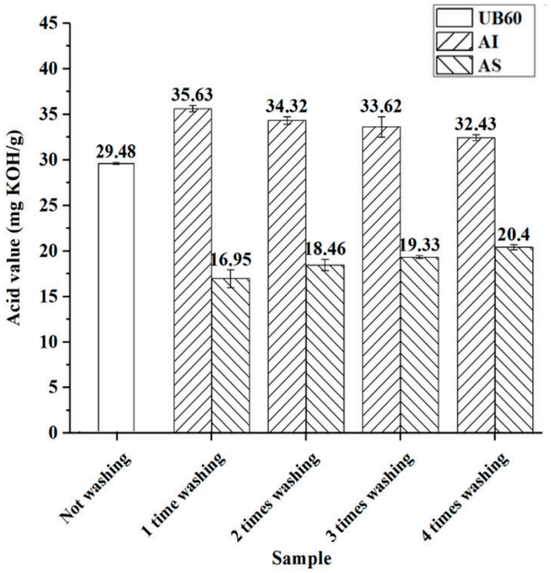

Figure 2. Acid values of main components of phospholipids

The acid values of AS and AI components obtained after different washing times were further determined. The results are shown in Figure 2.

The acid value of concentrated soybean phospholipid mainly comes from the acidic groups of phospholipid molecules and FFAs in non-phospholipids. Figure 2 shows that the acid value of UB60 is 29.48 mg KOH/g, the acid values of phospholipid component (AI) obtained after acetone washing are significantly higher than that of non-phospholipid component (AS). It indicates that the acid value of concentrated soybean phospholipid is mainly caused by phospholipid component, and the content of fatty acid is not high. With the increase of acetone washing times, the acid value of AI component gradually decreased, while the acid value of AS component increased. This phenomenon indicates that the free fatty acids in soybean phospholipids would gradually dissolve in acetone and eventually enrich in AS.

Iodine value of AS and AI

The higher the unsaturation degree of oil, the easier oxidation occurs. The iodine value is often used as an index to measure oil unsaturation degree. The iodine values of AS and AI in concentrated soybean phospholipid were determined. The results are shown in Figure 3.

Figure 3 shows that with the increase of acetone washing times, the iodine value of phospholipid component (AI) decreases continuously. The iodine value of unwashed concentrated soybean phospholipid (UB60) is 91.93 g I_2/100 g after four times of washing, the iodine value of AI component (PL4) decreases to 71.63 g I_2/100 g, while the average value of AS component (the iodine values of AS-40 and AS-80 is about 126 g I_2/100 g) is 126.18 g I_2/100 g, which was close to that of soybean oil (128.66 g I_2/100 g).

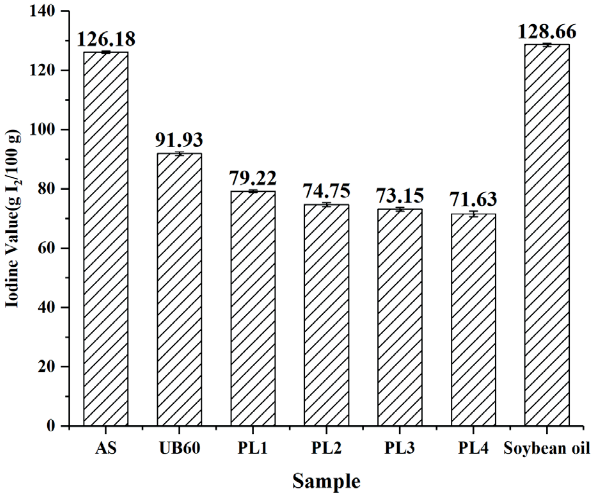

Figure 3. Iodine values of different phospholipids

The unsaturation degree of concentrated soybean phospholipids is high, and the iodine values of the AS and AI components are quite different. Therefore, the effect of the main components of phospholipids on the properties of phosphatidylcholine fatliquoring leather should be further investigated.

Effect of different components on leather properties

Phospholipids can influence the physical characteristics of leather, such as softness and wetting properties, but leather made with phospholipid fatliquors is prone to problems such as yellowing, elevated hexavalent chromium content, and undesirable odor in its storage, transport and use process. High unsaturation of concentrated soybean phospholipid and its free fatty acids, pigments, soybean oil and other factors may be the primary sources for the abovementioned problems. Therefore, different phospholipid components are compounded into different fatliquors according to the same formula, and the hexavalent chromium content, yellowing resistance and odor change of leathers fatliquored with those fatliquors were tested.

Effect of different components on hexavalent chromium content

The autoxidation of lipids in leather will produce hydroperoxides and so on. These substances are very active and have strong oxidizability, which can oxidize the free Cr (III) to Cr (VI).[29-30] In order to explore the influence of the main components of soybean phospholipid on the formation of hexavalent chromium, the hexavalent chromium contents in fatliquored leathers were determined after storage (7 days at room temperature) and dry heat treatment (80°C, 12 h). The results are shown in Figure 4.

Figure 4 shows that no Cr (VI) has been detected in the control sample (a synthetic emulsifier with saturated fatty alcohol as raw material, used for compounding phospholipid fatliquor), UB60 and AS-40 (distilled acetone at 40°C) fatliquored leathers after one week storage at room temperature. The hexavalent chromium content in the control sample after dry heat treatment is only 1.67 mg/kg. High hexavalent chromium content was detected in purified phospholipid (PL1-PL4) and AS-80 (distilled acetone at 80°C) fatliquored leathers after one week storage and dry heat treatment. Among them, the hexavalent chromium content in PL4 fatliquored leather was the highest, which is more than 110 mg/kg after one week storage or dry heat treatment. Figures 1 and 4 shows that with the increase of acetone washing times, the increase of phospholipid content (AI) and the decrease of non-phospholipid content (AS) in the samples (PL1-PL4) can lead to the increase of hexavalent chromium content. This phenomenon indicates that the improvement of phospholipid purity cannot solve the problem of elevated hexavalent chromium content.

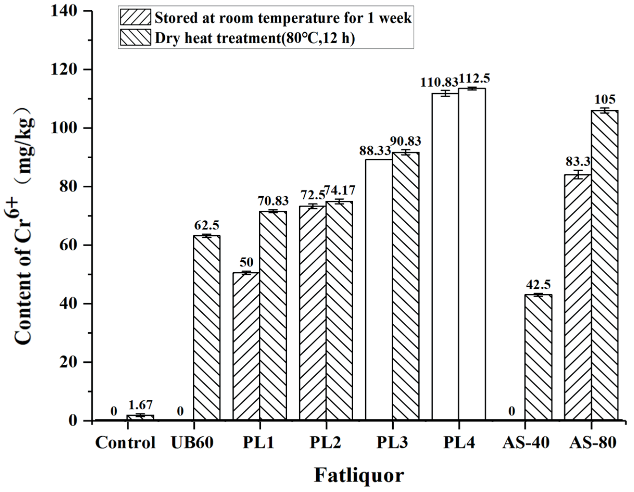

Figure 4. Hexavalent chromium content

The oxidation of the fatliquor is one of the main reasons for the hexavalent chromium increase. The hexavalent chromium content can reflect the oxidation degree of fatliquor in leather. The unsaturation of phospholipid is high. After four times of acetone washing, the iodine value of PL4 with 95% phospholipid content is still above 71 g/100 g (Figure 2). Therefore, the removal of non-phospholipid components (AS) in concentrated soybean phospholipid by acetone washing cannot solve the oxidation problem of phospholipid fatliquor.

It is worth mentioning that the content of non-phospholipid component (AS) in UB60 (38%) is much higher than that of PL4 (5%) (Figure 1), and the iodine value of UB60 is also higher than that of PL4. After one week storage and dry heat treatment, the hexavalent chromium content in the UB60 fatliquored leather is significantly lower than that in the PL4 fatliquored leather. This phenomenon indicates that the antioxidant activity of concentrated soybean phospholipid is higher than that of purified phospholipid. In addition, although the iodine value of AS is significantly higher than that of UB60 and PL1-PL4 (Figure 4), the AS-40 (distilled acetone at 40°C) has the strongest antioxidant activity. No hexavalent chromium was found in the AS-40 fatliquor leather after one week storage at room temperature. Even after dry heat treatment, the hexavalent chromium content in that is the lowest. However, the oxidation resistance of AS-80 (distilled acetone at 80°C) decreased significantly, and the hexavalent chromium content in the AS-80 fatliquored leather is significantly higher than that of AS-40 and UB60 fatliquored leathers. The reason for the above phenomenon may be that concentrated soybean phospholipid contains natural antioxidants. With the increase of acetone washing times, although the purity of phospholipid increases and its iodine value decreased, the antioxidants were dissolved by acetone. Therefore, the antioxidant activity of purified phospholipid becomes less, and the antioxidant activity of AS is enhanced with the increase of antioxidant concentration. After high temperature distillation at 80°C, the antioxidants in AS-80 destroyed and decomposed, resulting in the oxidative stability of AS-80 was significantly lower than that of AS-40. The existence and role of antioxidants in phospholipids will be discussed in detail in the following section.

Table I

Odor scores of fatliquored leathers stored at room temperature for 1 week

Sample	Evaluator No.1	No.2	No.3	No.4	Average score
Control	1	2	1	2	1.5
UB60	1	2	1	2	1.5
PL1	6	6	6	5	5.8
PL2	6	6	5	6	5.8
PL3	6	7	6	6	6.3
PL4	9	8	8	7	8
AS-40	1	1	1	1	1
AS-80	9	7	9	9	8.4

Note: 1 point = no peculiar smell; 5 = moderate odor; 9 = strong odor

Effect of different components on leather odors intensities

The oxidation of oil in leather can produce small molecule volatile substances, which can make leather confer offensive odors. It is one of the most serious problems of leather products. As mentioned above, the undesirable odor is a common problem of phospholipid fatliquors. As shown in Table I, the odor intensities of the fatliquored leathers after one week storage are different. The UB60 fatliquored leather has a slight smell (beany smell). The phospholipids (PL1-PL4) fatliquor leather purified by acetone washing have moderate undesirable odors, and with the increase of acetone washing times the odor of leathers is stronger, because the antioxidants in concentrated soybean phospholipid were washed out after acetone washing. The AS-40 fatliquored leather also has a slight smell, while the AS-80 fatliquored leather has a strong undesirable odor, which is due to the high unsaturation of AS, and the antioxidants decompose at high temperature (80°C). Figure 4 and Table I shows that odor sensory and hexavalent chromium test data show a strong correlation. The higher the hexavalent chromium content, the higher the odor concentration.

With the prolongation of storage time, the peculiar smell of leathers was aggravated. After one month storage, UB60 and AS-40 fatliquored leathers conferred offensive odors (5 points); The oiliness, softness of leather decreased significantly. As mentioned above, natural antioxidants in UB60 and AS-40 delay the oxidation of the lipids.

The following experiments were planned to further confirm that the odor in leather was caused by oil oxidation. The volatile compounds in the control sample and AS-80 fatliquored leathers were collected and studied by HS-SPEM-GC-MS. The volatile compound mass spectrum data was automatically vetted through the atlas library and the components with low content and low confidence were discarded. Lastly, the relative percentage content of volatile compounds was determined by gas chromatography peak area percentage method.

Through GC-MS analysis found that there is a significant difference in the composition of volatile gas between the control sample and AS-80 fatliquored leathers. As shown in Table II, 41 volatile substances were detected in the control sample, and 67 substances were detected in the AS-80 fatliquored leather. Although there are 32 types of the same substances in the two kinds of fatliquored leathers, there are 29 types of aldehydes, esters, ketones and ethers (their thresholds are low) in the volatile components of the AS-80 fatliquored leather, and the relative total content is 45.94%. The types and relative contents of that volatile components in the AS-80 fatliquored leather are higher than those in the control samples (9 kinds, 37.56%).

The volatile compounds produced by oil oxidation mainly include aldehydes, ketones, acids, esters, furans, alcohols and hydrocarbons. The content of linoleic acid in soybean oil and phospholipids is about 50%, and aldehydes are mostly the oxidative degradation products of linoleic acid. The odor characteristics of saturated aldehydes are related to concentration. The odor threshold of aldehyde is very low. The odor characteristics of saturated aldehydes are related to concentration. When the concentration is low, it will present a sweet and fruity odor, and when the concentration is high, it will produce a pungent odor.[31] Table III shows the types and contents of volatile aldehydes in the control sample and AS-80 fatliquored leathers. It can be seen from Table III that the types of aldehydes in the AS-80 fatliquored leather are obviously more than that in the control sample fatliquored leather. In addition, the contents of trans-2-octenal, trans-2-decenal, nonanal and N-octyl aldehyde (these substances are the typical odiferous substances of oil oxidation.) in the AS-

Table II

Types and relative contents of volatiles in leathers

		alcohol	ester	ether	ketone	alcohol	alkane	others	total
Control sample	types	3	4	1	1	12	17	3	41
	Relative content %	3.22	30.32	1.53	2.49	43.79	15.48	2.79	99.62
AS-80	types	13	9	2	4	17	19	4	67
	Relative content %	17.96	23.14	1.00	3.84	38.14	11.89	2.08	98.05

Table III
GC-MS detection of main volatile aldehydes

No.	Name of components	Relative content %	
		Control sample	AS-80
1	Trans-2-decenal	-	3.15
2	Trans-2-octenal	1.39%	3.25
3	Nonanal	1.47%	3.18
4	2- undecylenic aldehyde	-	2.03
5	Trans (E)-nonenal	-	1.99
6	(E, E)-2, 4-nonadienal	-	1.37
7	N-octyl aldehyde	-	1.13
8	Trans-4, 5-epoxy-(E)-2-decanal	-	0.64
9	(2E)-2-hexadecenal	-	0.36
10	4-ketoaldehyde	-	0.30
11	Benzaldehyde	-	0.28
12	7, 11-hexadecadienal	-	0.28
13	2-heptanal	0.36%	-

80 fatliquored leather are higher than that in the control sample fatliquored leather. Therefore, the volatile aldehydes produced by oil oxidative rancidity in the fatliquor is the main reason for the odor increase in the fatliquored leather.

(α) Tocopherol Content and Antioxidant Activity of (α) Tocopherol

Figure 4 shows that there are antioxidant substances in phospholipids which can inhibit phospholipid oxidation, thus reducing the hexavalent chromium. Among them, tocopherol (VE) is the most important antioxidant in soybean phospholipids.[32] Thus representative natural antioxidant (α) tocopherol became a research focal point. The content of (α) tocopherol in the main components of phospholipids was detected, and its antioxidant properties to phospholipids were investigated.

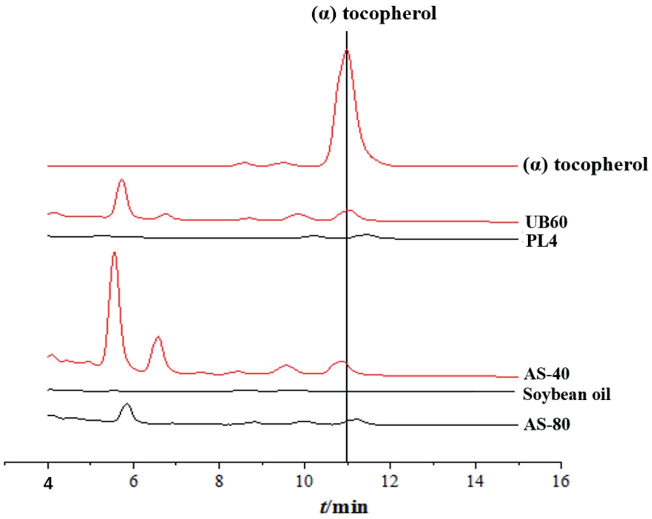

Figure 5. (α) Tocopherol Liquid Chromatogram curve

Table IV
(α) tocopherol contents in samples

Sample	Content (mg/g)
UB60	0.064
PL4	0
AS-40	0.159
AS-80	0
Soybean oil	0

Figure 5 shows that (α) tocopherol appears in both UB60 and AS-40, but not in PL4, AS-80 and soybean oil. (α) tocopherol is not resistant to high temperature. After long time distillation at 80°C, (α) tocopherol in AS was decomposed and destroyed.[32-34] Therefore, the presence of (α)tocopherol is almost undetectable in AS-80.

The concentration of (α) tocopherol was further determined. It can be seen from the results in Table IV that the (α) tocopherol concentration in AS-40 (0.159 mg/g) is much higher than that in UB60 (0.064 mg/g), while the tocopherol contents in PL4, soybean oil and AS-80 are 0. This phenomenon indicates that there is tocopherol in concentrated phospholipid (α) tocopherol, after repeated acetone elution, (α) tocopherol, a fat soluble substance, will flow out with acetone and finally enrich in AS.

Figure 4 and Table IV shows that the (α) tocopherol content in phospholipids and hexavalent chromium test data show a strong correlation. The presence of (α) tocopherol can inhibit the oxidation of phospholipids obviously. Therefore, adding antioxidants to phospholipids is an effective way to solve the problem of hexavalent chromium and peculiar smell caused by oxidation of phospholipid fatliquors. The antioxidant effect of (α) tocopherol on phospholipid fatliquor was further investigated. It was found that when 1% (α) tocopherol was added to UB60, the UB60 fatliquored leather had no undesirable odor after one week, but still appeared a strong undesirable odor after one month. After dry heat treatment (80°C, 12 h), the hexavalent chromium content of leather was still as high as 58.3 mg/kg, which indicated that (α) tocopherol was not strong in improving the antioxidant ability of soybean phospholipid. In addition, (α) tocopherol is not resistant to high temperature. Therefore, it is necessary to further optimize the antioxidant.

Effect of phospholipid saturation on leather properties

It can be seen from the previous experimental results that tocopherol can effectively reduce but not completely prevent the formation of hexavalent chromium caused by oxidation of phospholipid. The

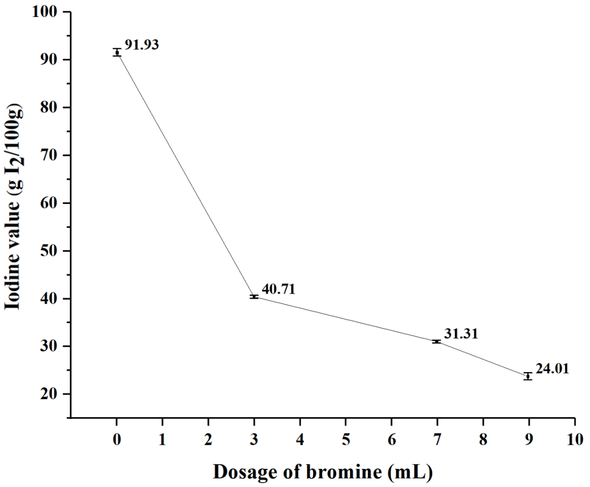

Figure 6. Iodine values of modified phospholipids

high unsaturation is the main reason for the low oxidation stability of soybean phospholipids. Therefore, bromine was used to modify UB60 in different degrees, and the effect of phospholipid saturation on the properties of fatliquored leathers was investigated.

The iodine value of bromine-modified phospholipids in different degrees

Different amounts of bromine were used for the addition reaction of 15 g UB60 to obtain phospholipids with different saturations. The iodine values are shown in Figure 6.

Figure 6 shows that the iodine value of phospholipid after brominated modification decreases with the increase of bromine dosage, but the bromine dosage is not completely proportional to the decrease of iodine value of phospholipids. The main reason is that not only the addition reaction but also the substitution reaction will occur when bromine is added to the phospholipid.

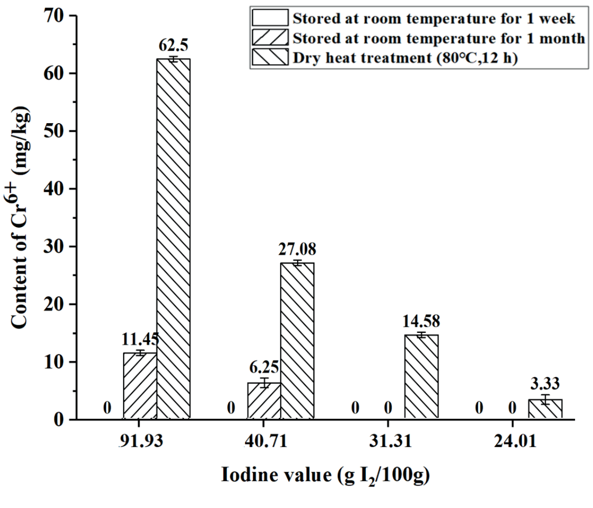

Figure 7. Hexavalent Chromium Content in modified phospholipid fatliquored leathers

Effect of phospholipid saturation on hexavalent chromium

The hexavalent chromium content in leather fatliquored with phospholipids with different Iodine value after storage (7 days and 1 month at room temperature) and dry heat treatment (80°C, 12 h) is shown in Figure 7.

Figure 7 shows that there is no hexavalent chromium in the leathers after one week storage; After one month storage, the hexavalent chromium contents in the leathers fatliquored with phospholipid fatliquors with high iodine value (about 92 and 41 g I_2/100 g) were 11.45 and 6.25 mg/kg respectively; However, no hexavalent chromium was found in the leathers fatliquored with phospholipid fatliquors with low iodine value (less than 32 g I_2/100 g); After dry heat treatment (80°C for 12 h), the hexavalent chromium content decreased with the decrease of phospholipid iodine value; It is worth noting that when the phospholipid iodine value was reduced to about 24 g I_2/100 g, the phospholipids had excellent antioxidant properties, and the hexavalent chromium content is only 3.33 mg/kg after dry heat treatment.

The odor of different modified phospholipid fatliquored leathers during storage was evaluated. It was found that the leathers with phospholipid fatliquors with the lowest iodine value had no undesirable odor after one month storage, while the others had different degrees of undesirable odor.

According to the above results, it can be concluded that the high unsaturation of soybean phospholipid is the main reason for poor oxidation stability of soybean phospholipid. The antioxidant activity of phospholipids could be enhanced with the decrease of unsaturation of phospholipids. When the iodine value of phospholipid is reduced to about 20 g I_2/100 g, the problem of elevated hexavalent chromium content and undesirable odor can be completely solved.

Effect of phospholipid saturation on yellowing resistance

Yellowing resistance is an important index to evaluate leather products, and fatliquor is one of the main factors affecting the yellowing of leather. Oil, pigment and other substances can accelerate oxidative browning under light and high temperature conditions, resulting in yellowing of leather. The light and heat yellowing resistance of leathers fatliquored with several phospholipid fatliquors with different iodine values was further studied. The color difference values of leather before and after illumination (300 W xenon lamp, 50°C, 24 h) and dry heat treatment (120°C, 24 h) were measured by a colorimeter, and ΔE (comprehensive deviation of color difference) represents the yellowing degree of leather.

It can be seen from Table V that with the decrease of modified phospholipid iodine value, the light-resistance and heat-resistant yellowing properties of fatliquored leather are improved, especially

Table V

ΔE of modified phospholipid fatliquored leathers

Iodine value ($g\ I_2/100g$)	ΔE	
	Illumination (50°C, Xenon lamp, 24 h)	Dry heat treatment (120°C, 24h)
91.93	1.46	10.97
40.71	1.37	5.86
31.31	1.36	3.25
24.01	1.29	2.58

the heat-resistant yellowing properties. The heat-resistant yellowing was significantly enhanced with the decrease of iodine value of modified phospholipid. The color difference value of unmodified phospholipid (UB60) fatliquor leather before and after dry heat treatment is as high as 10.97, and the leather appearance is seriously yellowing, and the handle is dry and hard. The heat-resistant yellowing ability of the leather with modified phospholipid (Iodine value= 24.01 g I_2/100 g) fatliquors is the best, and the color difference value before and after dry heat treatment is only 2.58, the leather can still maintain good softness.

Conclusion

Leather made with soybean phospholipid fatliquors is prone to problems such as yellowing, elevated hexavalent chromium content, and undesirable odor, which are mainly caused by the oxidation of phospholipid. The volatile aldehydes produced by lipid oxidative rancidity are the main components of the undesirable odor. The purification of natural soybean phospholipid through simply removes the non-phospholipid components cannot solve the problems caused by oxidation of phospholipid. as a typical natural antioxidant existing in natural soybean phospholipid, tocopherols can restrain the oxidation of phospholipid to a certain degree, however, the dissolving out and destruction of tocopherols at high temperature in the phospholipid purification process can lead to more obviously oxidation of phospholipids. Additionally, the oxidation defects of phospholipid cannot be completely resolved by adding extra tocopherols, even at high dosages. The research finds that the defects of soybean phospholipid fatliquors can be thoroughly solved by increasing the saturation degree of lipid through addition reaction, the suggested iodine value of phospholipid products is lower than 20 g I_2/100 g.

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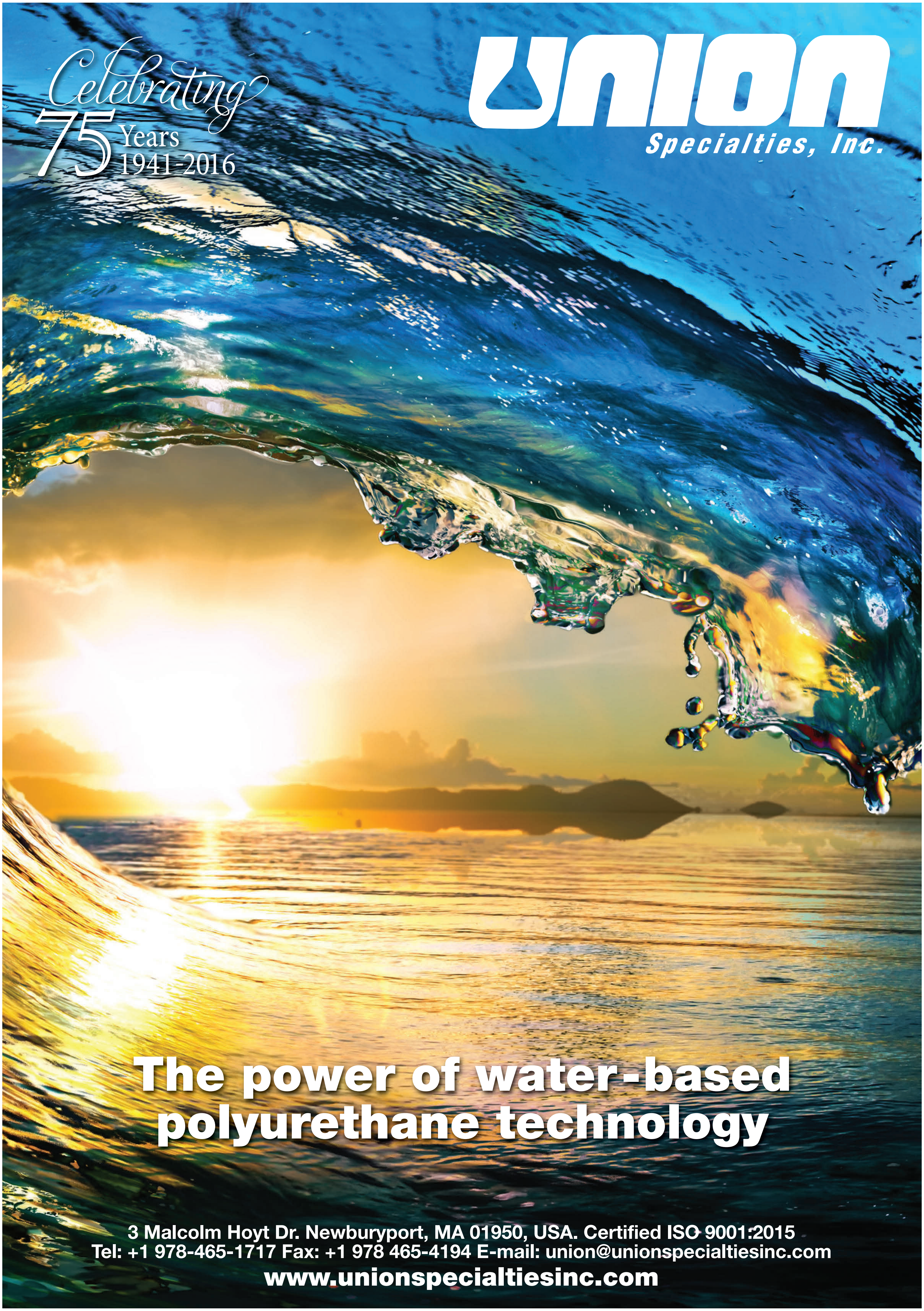

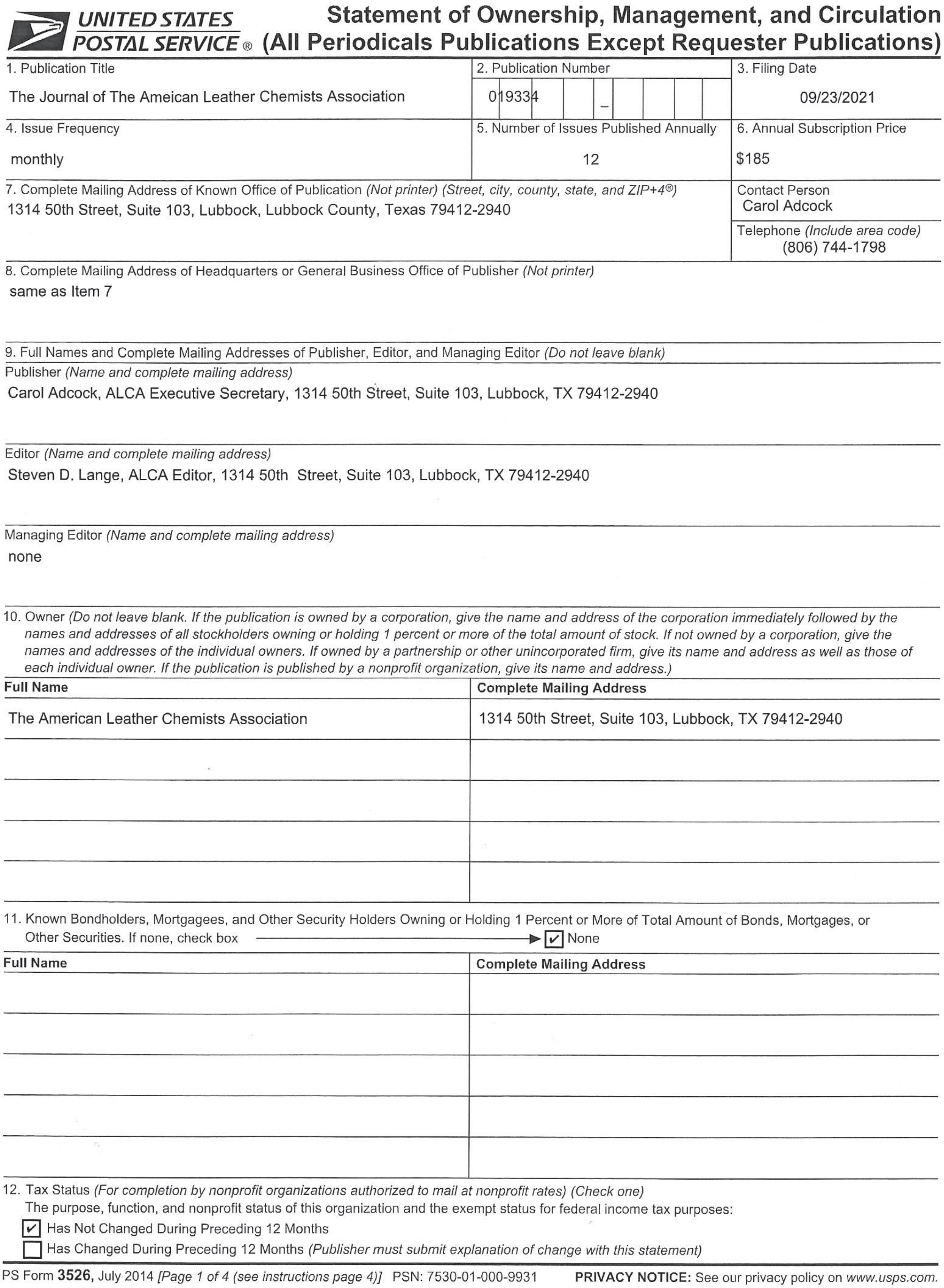

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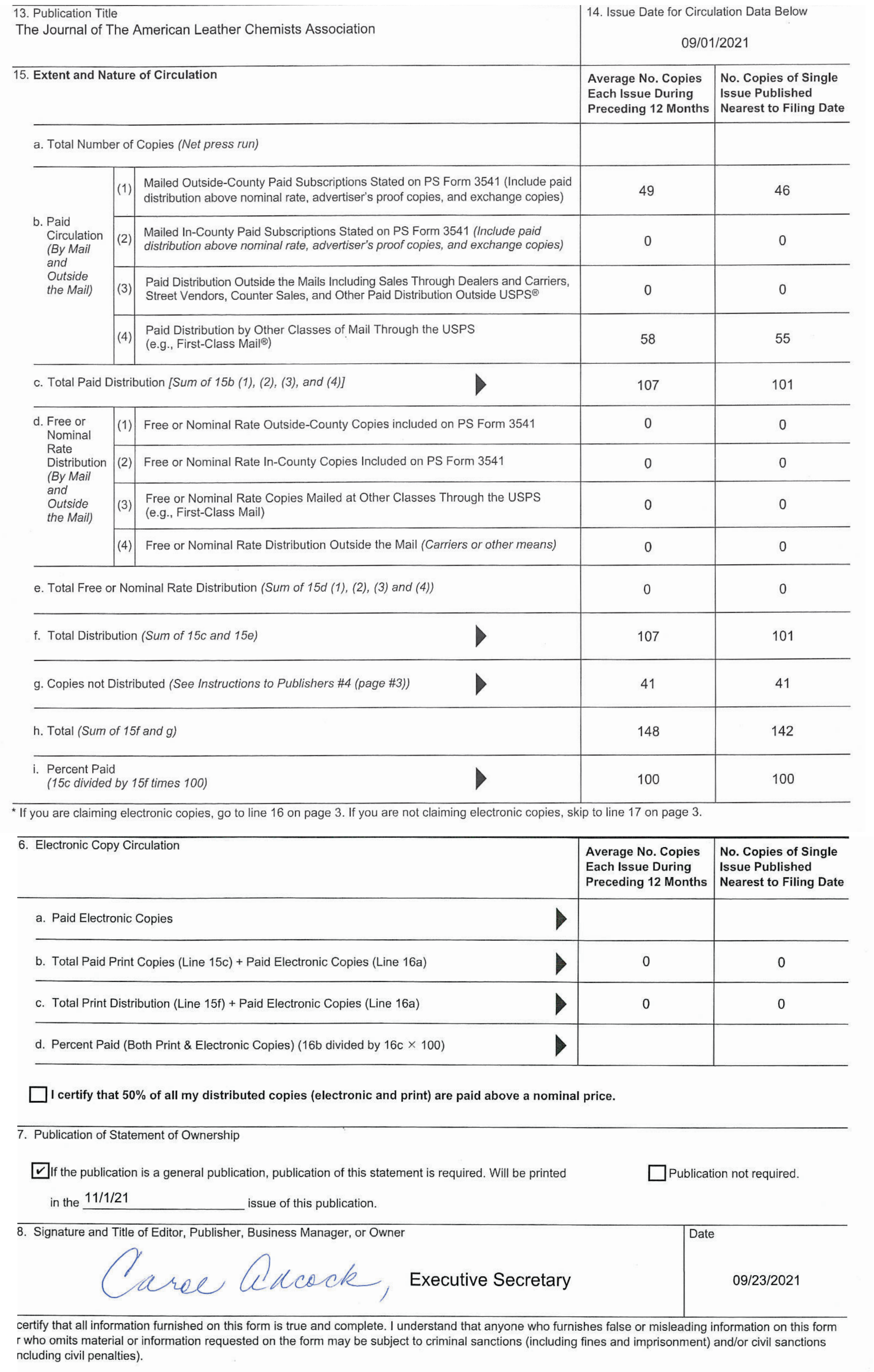

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Lifelines

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Chemtan	*378*
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