Paper B8

Studies on role of unnatural amino acids in tanning process for making chrome-free leather

Ganesan Krishnamoorthy[1], Sayeed Sadulla[*,2], Praveen Kumar Sehgal[1] and Asit Baran Mandal[1]
[1]CSIR - Central Leather Research Institute, Adyar, Chennai-600 020, Tamil Nadu, India
[2] Indian Shoe Federation, Egmore, Chennai-600 008, Tamil Nadu, India,
Corresponding author E-mail: sadullams@hotmail.com

Abstract: In the present study, green method or eco-friendly approach to tanning processes based on unnatural D-amino acids (D-AA)-aldehyde (Ald) as a substitute for chrome-free tanning has been attempted. The distribution of optically active D-AA in tanned leather, the hydrothermal stability, the mechanical properties, resistance to collagenolytic activity and the anti-microbial properties of tanned leather as well as the evaluation of eco-friendly characteristics were investigated. Scanning Electron Microscopic (SEM) and Atomic Force Microscopic (AFM) analysis indicate that the surface morphology and roughness of the tanned leather collagen matrix are even when the D-AA concentration is lower than 5 wt% (on bated pelt weight). Differential Scanning Calorimetric (DSC) analysis shows that the shrinkage temperature (Ts^o) of tanned leather which is related to the content of D-AA is more than that of Ald tanned leather or equal to chrome tanned leather. This D-AA tanned leather demonstrates a higher resistance to microbial activities than conventional wet blue leather. The latter incubated for 3 days began to show microbial growth; while the unnatural D-AA tanned leather which was incubated for 6 days had no microbes, showing significant anti-microbial effect. Environmental Impact Assessment (EIA) shows that the developed process results in significant reduction in total solids (TS) and better biodegradability of organic compound in the effluent when compared to chrome tanning.

Key words: Unnatural D-amino acids; Combination tanning; Chrome-free tanning.

1. Introduction

Originally, leather production is an eco-friendly industry, because slaughter house wastes viz the hides/skins discarded as waste material are processed into useful materials such as leather goods, footwear and garments. During the leather making process, tanning is one of the most important operations, which improve the durability and practicability of leather products and prevent putrefaction, in which the tanning agents react with the collagen molecule, stabilizing the triple helical structure of collagen matrix; thereby the leather acquires resistance towards chemical, thermal and microbiological degradation.

Usually the tanning process primarily employs inorganic tannins such as chromium, aluminium, titanium, iron and zirconium etc., and organic tannins such as aldehyde and high molecular weight vegetable tannins (hydrolyzable and condensed), synthetic tannins and above combinations. However, each of above tannins has some advantages and disadvantages. The disadvantages of the known tanning agents include their limited availability and their minimal recoverability or reusability from leather waste. Wastewaters are difficult to be treated with conventional treatment systems.

Conventional tanning processes include the use of chrome and other inorganic agents, which transform the perishable raw hides into durable leather, but at the expense of our natural resources. Chrome tanning is the most widely used tanning process in the industry, for performance, economic and ecological reasons. Even though state of the art tanneries do succeed in reducing the chromium contents of their effluents to below the stipulated maximum values, this is only possible at enormous expense. On the other hand, the quality and variety of leathers obtained by chrome tanning must be

considered extremely high, so that to date no other tanning processes have been found that provide the same universality, quality and variety of styles. The chrome and other inorganic pollutants contaminate our air and water. The chrome-tanned leather sometimes generates certain hazardous substances and then it is cytotoxic in human health and it pollutes environment. Leather waste hexavalent chromium is potentially hazardous to human health and a new technology of its treatment has been reported [1,2].

Vegetable tannins, natural materials, have been considered as a suitable eco-friendly option to replace chromium and vegetable tanning is being employed for making some kinds of leathers. However, this tanning leads to excessive loading in the leathers, which reduces its versatility to make different end products and also has low resource availability. This vegetable tanned leather is also known to be poorly biodegradable, which results in high Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD).

Hence, the combination tanning systems were considered as suitable tanning method to overcome the problems from single tanning system. Various combination-tanning exist in the world. Presently, the combination tanning are mainly based on chrome-less and chrome-free tanning processes. For examples, less chrome combination tanning systems are chromium and silica, chromium and iron, etc. The chrome-free combination tanning systems based on organic such as vegetable with synthetic tannin and inorganic materials such as metal ions other than chromium have been reported [3-9]. However, all these above combination tanning do not have commercial importance in the global leather industry due to processing difficulty, toxicity, availability and cost, etc. The elimination/reduction of chrome and other inorganic substances assume importance in the tanning process in present conditions. The 21st century has embraced the appearing of green chemistry era and an up-coming that has attracted the attention and participation of many researchers resulting in many new reactions, new processes, and new commercial possibilities and processes that minimize the use and generation of hazardous substances. The importance of green chemistry process in research and development for industrial application for minimization of the use and generation of hazardous substances have been reviewed [10-12].

In this present study, the development of eco-friendly tanning based on optically active unnatural D-AA has been attempted as an alternative chrome-free tanning. The tanning conditions of D-AA, physical-chemical characteristics of leathers and the environment impact of this tanning have been compared with conventional tanning processes.

2. Materials and methods

2.1. Materials

Raw material used for leather processing was pickled goat skins. The chemicals employed in subsequent operations were those normally used in leather industry. All the other chemicals were commercial grade products.

2.2. Preparation of unnatural D-AA

L-Alanine (L-Ala) racemization was performed using a 30% solution of L-Ala hydrochloride with 25% strength NH_3 solution for 5 hours at 70° C in an autoclave. The discharged mixture was brought to pH 6.1 with conc. HCl and salts were removed by electrodialysis. Subsequent crystallization by evaporating down the aqueous solution gave 70 % of pure D-Alanine (D-Ala).

L-Glutamic acid (L-Glu) racemization was performed using a 30% solution of L-Glu hydrochloride. This solution was stirred at 135°C for 105 h and added 10% of aniline. To the resulting solution, 800 ml ethanol and allowed to stand overnight in the refrigerator. The crystalline D-Glutamic acid (D-Glu) is filtered off and washed with 95% ethanol until free of chloride. The yield is 95 %.

L-Lysine (L-Lys) racemization was performed using a 30% solution of L-Lys hydrochloride. A 200 ml solution in a 300-ml autoclave was stirred at 135°C for 105 h. To the resulting solution, 800 ml ethanol was added to crystallize-out D-Lysine (D-Lys). The yield is 90 %.

2.3. Tanning and shrinkage temperature determination

4 pickled goat skins were tanned by 5 % (based on pickled weight) unnatural D-AA (D-Ala, D-Glu and D-Lys) with 1% glutaraldehyde (GA), for 3 h and then the basification was carried out by the addition of 1 % sodium formate and 1 % sodium bicarbonate in four instalments at an interval of 15 min. Finally, the tanning drum was run for 1 h and Ts^o of each tanned leathers were determined.

2.4. Characterization

The mechanical and thermal properties and morphological changes of unnatural D-AA tanned leathers were analyzed using TMA (Instron series II Automated Materials Testing System), DSC (TA-DSC Q 200) SEM (FEI QUANTA 200) and AFM (Park Systems XE-70) analysis.

The % of degradation of D-AA tanned leather against collagenases activity was calculated by an earlier method [13]. The antimicrobial activity was tested by disc diffusion method.

The D-AA tanned leather liquor from experimental processing was collected and analyzed for BOD and COD. TS was calculated according to the standard procedure. The values reported are average of 3 experiments along with their standard deviations.

3. Results

Interest in stereoisomer of optically active unnatural D-AA has increased attention in recent decades with the development of new analytical methods. Incorporation of unnatural D-AA improves the stability to protein, from a functional point of view; their unnatural stereochemistry often means that compounds containing D-residues are much more resistant to enzyme-catalyzed breakdown than natural peptides, a property of considerable pharmaceutical, food and microbiologic importance [14,15]. These are of profound interest to establish how the optically active D-AA assisted tanning process in order to improve the stability and resistance to collagenolytic activity.

3.1. The hydrothermal stability

The denaturation temperature (T_D) of unnatural D-AA+Ald tanned leather was studied using DSC analysis. The position, width, height and symmetry of the thermogram peak provide information about the thermal denaturation of leather over a defined temperature range. The Ts^o of leather is normally related to the onset temperature of its peak in a DSC pattern and can be used as a measure of the hydrothermal stability of tanned leather subjected to different D-AA treatments are shown Table I.

Table I. Hydrothermal stability, shrinkage temperature and mechanical characteristics

Processes	T_D (^oC)	Ts^o (^oC)	TS (Mpa)	% E
BCS	120±3	104±3	18	56±3
Veg	103±2	83±2	15	67±5
Ald	110±5	90±5	15	72±6
D-Ala+Ald	105±3	96±3	14	67±3
D-Glu+Ald	101±7	92±4	15	52±6
D-Lys+Ald	120±7	120±3	18	56±6

An increase in the Ts^o is usually an indication of an increase in the stability of wet tanned leather. Fortunately, hide treated with D-Lys+Ald, the endothermic peaks are similar to chrome (BCS) tanned leather and become narrow and sharp, moreover, the Ts^o of final leather reach 104, 83, 90, 96, 92 and 120, respectively (Table I). Obviously, the combination tanning can efficiently improve the

hydrothermal stability of leathers and the tanning effects of D-Lys+Ald are superior to Ald and Veg tannin. The D-Lys+Ald tanned leathers were found to be soft, full and possessed tight grain; these leathers, having better hydrothermal stability. This could be possible possibly due to the decrease in the availability of the active sites in the leather matrix for interaction of D-Lys after treatment with Ald. The Ts^{o} of fibrillar collagens depends on stable intermolecular cross-links formed through the Lys Ald.

3.2. Mechanical properties

Tensile strength (TS) and % Elongation (% E) of D-AA tanned leather were determined and compared to BCS and Vegetable tanned leather (Table I). The mean values corresponding to each experiment are given in Table I. The D-Lys+Ald tanned leather are close to the BCS tanned leather. D-Lys+Ald tanned exhibited high TS and low % E where as D-Ala+Ald and D-Glu+Ald tanned leather exhibited low Ts with high % E. And this is due to the fact that the fibre bundles are well separated in the case of D-Ala+Ald and D-Glu+Ald tanned leather, while D-Lys-Ald tanned leather shows cemented fibres bundles as revealed by SEM studies.

3.4. AFM analysis of grain surface roughness

Fig. 1 shows the plane and three-dimensional profiles of the tanned leather grain surfaces. Lighter areas of the images correspond to higher topography and darker areas correspond to lower topography. D-AA assisted tanned leather has been attempted for the first time in this present study. Ald tanned leather shows a rougher texture with higher peaks than BCS and vegetable tanned leather.

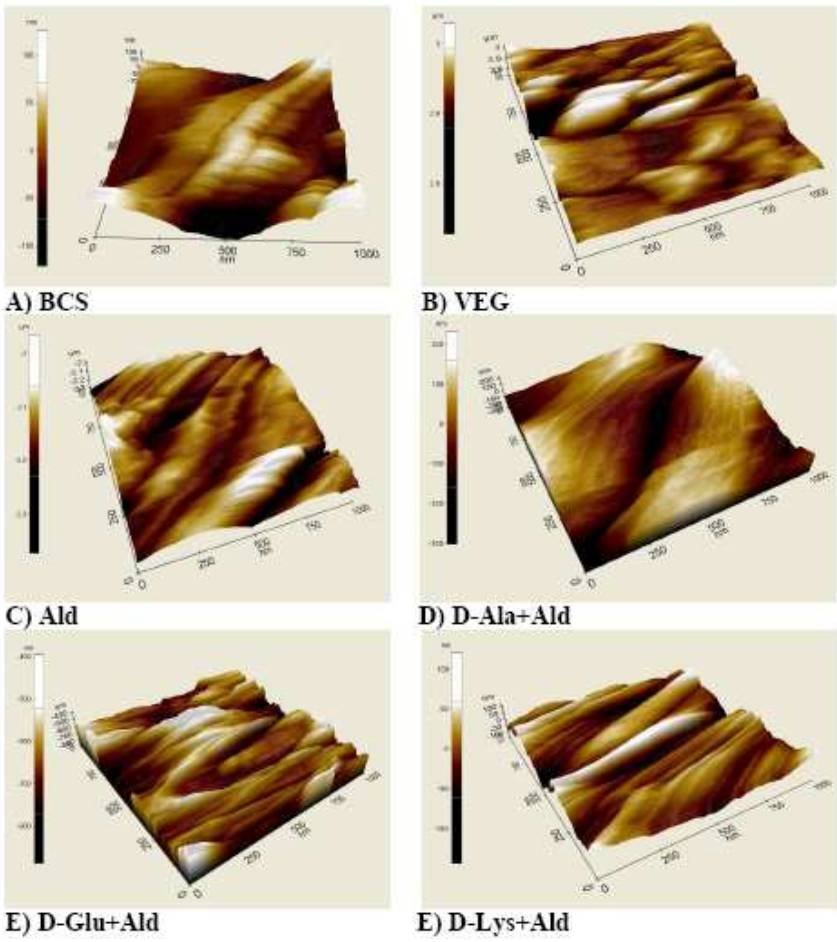

Fig. 1. AFM analysis of D-AA tanned leather

Tanning by D-AA is thought to be the reason for the increased stability of the tanned leather that is manifested by a higher Ts. Tanned leather clearly formed fibrils in the presence of D-AA as shown by the AFM images where the typical collagen banding pattern of grooves and ridges (Fig. 1). The width of the D-periodicity of collagen fibrils from tanned leather did not appear to be significantly different. Also, there was not a difference when the fibrils were formed in the presence or in the absence of D-AA. Measurements of collagen fibrils in BCS tanned leather (Fig. 1A) showed them to have a mean D-periodicity of 32 nm with a standard deviation of 9; measurements of VEG tanned leather (Fig. 1B) had a mean D-periodicity of 165 nm; ALD tanned leather (Fig. 1C) had a mean D-periodicity of 65 nm; presence of D-Ala (Fig. 1D) had a mean D-periodicity of 65 nm; presence of D-Glu (Fig. 1E) had a mean D-periodicity of 165 nm; and presence of D-Glu (Fig 1F) had a mean D-periodicity of 166 nm. These distances are not significantly different and are characteristic of the axial periodicity of collagen fibrils.

While the D-periodicity did not vary, neither was there a statistically significant difference between the widths of the fibrils. Measuring widths of fibrils is more difficult than measuring the D-periodicity and is also more subjective as the fibrils form overlapping networks where the sides of a fibril may not always be clearly determined. The fibril widths measured can therefore only be considered as approximate. Measurements of collagen fibrils in BCS tanned leather (Fig. 1A) showed that they had a mean width of 123 nm with a standard deviation of 20; Veg tanned leather (Fig. 1B) had a mean width of 82 nm; Ald tanned leather (Fig. 1C) had a mean width of 72 nm; D-Ala+Ald tanned leather (Fig. 1D) had a mean width 92 nm; D-Glu+Ald tanned leather (Fig. 1E) had a mean width 92 nm; D-Lys+Ald tanned leather (Fig. 1F) had a mean width 92 nm.

The ability of the collagen monomers to align in a quarter-stagger arrangement in the presence of D-Lys+Ald (Fig 1D) shows that the size of these molecules does not hinder the formation of collagen fibrils. Although it has been shown that D-Glu+Ald results in the formation of reactive adducts of some amino acid residues found in collagen, which in turn may result in the formation of inter or intramolecular cross-links between collagen molecules, it does not appear to affect the structure or the arrangement of the collagen fibrils.

A similar study performed with D-Lys+Ald complexes showed that though D-Lys helped form the quarter-stagger structure and induced a fibrous network of aggregated collagen molecules that lacked the D-periodicity, possibly because of size constraints. The presence of D-Lys with Ald during lateral assembly of collagen filaments decreased the rate of lateral disassembly thus helping a faster net rate of stable fibril formation. In our study with D-Lys+Ald, we do not observe an increase in collagen molecules lacking D-periodicity or a fibrous aggregated network. On the contrary, AFM analysis of collagen fibrils cross-linked with D-Lys+Ald revealed a well-ordered structure with the fibrils properly oriented and well aligned compared to collagen fibrils in the absence of D-Lys+Ald (Fig. 1F).

3.5. SEM analysis.

The crust leathers can be assessed by viewing the grain surface and cross section of leather samples using SEM. The SEM analysis of BCS and D-AA tanned leather samples showing grain surface are given in Fig. 2A, 2B, 2C, 2D, 2E and 2F.

All the D-AA+Ald tanned leathers exhibit a clear grain surface, which indicates that there is no physical deposition. Higher magnification (×1000) SEM [not shown] confirm the above observation, where the hair follicles look clean without any foreign materials in all cases. The grain surface of the leather tanned using D-AA+Ald seems to be flat without any wrinkles compared to the leather tanned with the BCS. This could be due to the rapid reaction of the D-AA+Ald with the grain surface as against a mild and gradual fixation of chromium in chrome tanning. The SEM analysis of D-AA tanned leather samples showing cross section a magnification of 500 are depicted are given in Fig. 3A, 3B, 3C, 3D, 3E and 3F. The D-AA tanned leather show the compactness in the fibres structure throughout the cross section indicating the uniform structure. In specific, D-Lys+Ald tanned leathers show more compact fibre structure compared to BCS. The fibre bundles seem to be less dispersed (separation of fibres) in the D-Lys+Ald tanned sample compared to a BCS tanned sample.

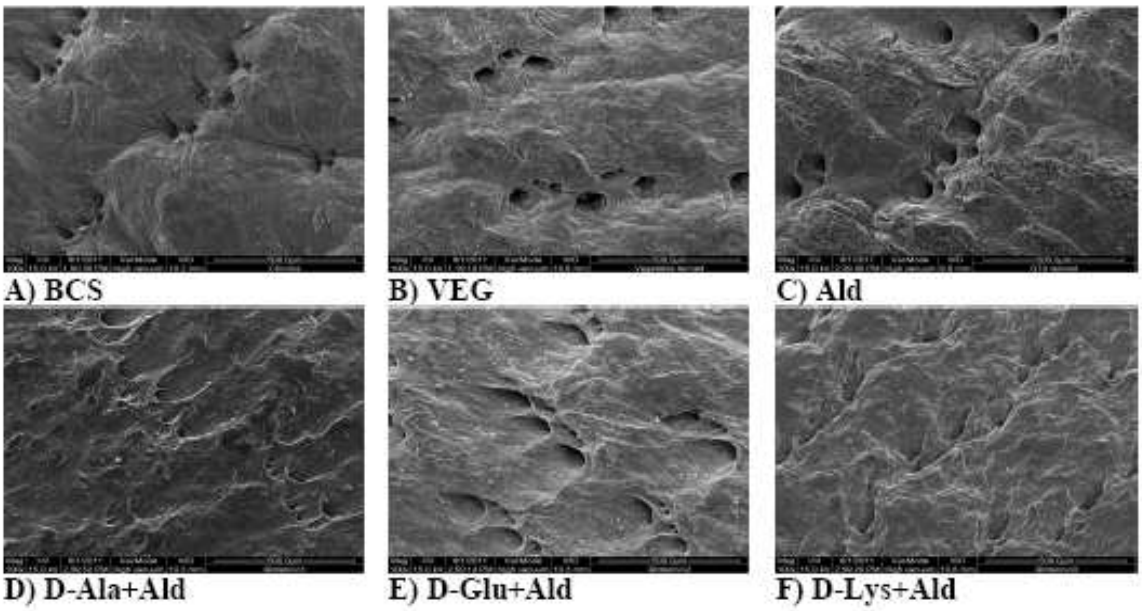

Fig. 2. SEM image of grain surface of D-AA tanned leather

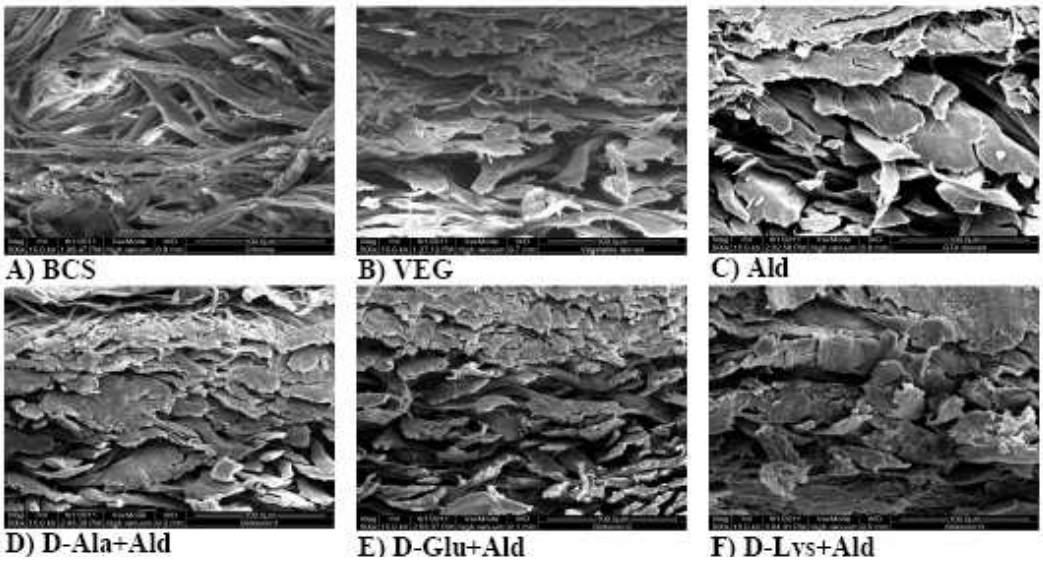

Fig. 3. SEM image of cross section of D-AA tanned leather

Since the D-Lys+Ald tanned sample exhibits slightly dispersed (opened up) fibre structure, it would, in principle, exhibit an increased fullness and softness in the final leather.

3.5. Biodegradation

% leather degradation (based on hydroxyproline released) for unnatural D-AA tanned leather by collagenases at various time periods has been determined (Fig.4).

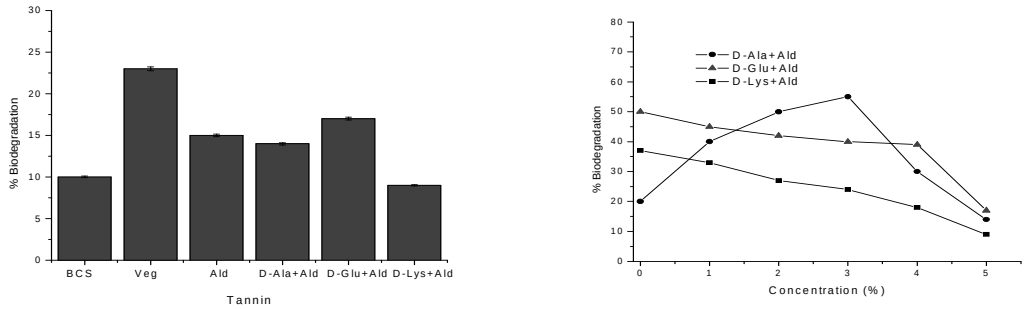

Fig. 4. The degradation of D-AA tanned leather by collagenases

Significant reduction in the degradation of leather is observed for D-AA tanned leather compared to chrome tanned leather. The D-Lys+Ald tanned leather exhibited 9 % degradation of leather as against 99% degradation in the case of untanned (pickled skin) native at 96 h period of incubation. The stability of D-AA tanned leather against collagenases would have been brought about by protecting the active sites in collagen through interaction with D-AA recognized by collagenases. The significant differences in the enzymatic stability offered by D-Lys+Ald could be due to the effectiveness of the latter in exhibiting better interaction with collagen through multiple inter and intramolecular crosslinks. Lysine and hydroxylysine Ald-derived crosslinking between and among collagen molecules are major elements in stabilizing fibrillar collagens.

3.6. The anti-microbial effect

In vitro antimicrobial study showing the activity of tanned leather against B. Subtilis, E. coli and S. areus are presented in Table II. The antibacterial activity of unnatural D-AA tanned leather determined by disc diffusion assay showed a zone of inhibition for B. subtilis (13, 12, 14 mm), E. coli (9, 12, 11 mm) and S. areus (14, 16, 18 mm) after 24 h and it was maintained for a period of 6 days.

Table II. Anti-bacterial activities of unnatural D-AA tanned leather

Process	Zone of inhibition (mm)								
	B. subtillis			E. coli			S. areus		
	1st	3rd	6th	1st	3rd	6th	1st	3rd	6th
BCS	-	-	-	-	-	-	-	-	-
Veg	-	-	-	-	-	-	-	-	-
Ald	-	-	-	-	-	-	-	-	-
D-Ala+Ald	3	7	13	3	6	9	5	7	14
D-Glu+Ald	4	6	12	3	7	12	6	8	16
D-Lys+Ald	6	9	14	4	8	11	7	9	18

It can be seen that the D-AA assisted tanned leathers which were incubated for 6 days had no growth, while the BCS tanned leather which was incubated for 3 days began to growth. This D-AA is very useful for biomaterials or leather-making. Hides which have been tanned with D-Lys+Ald demonstrate a higher resistance to microbes than conventional wet blue leather. Provided that the hides are further processed within a week, there is no need for treatment with a bactericide or a fungicide.

3.7. Environmental impact assessments

The impact of pickle less unnatural D-AA assisted tanning system on the environment was assessed by the spent tan liquor analysis for TS, BOD_5 and COD. It can be seen from Table III that the COD value in combination tanning is higher than in BCS tanning due to the applications of D-AA.

While the TS load value is lower than that of the BCS tanning process. And the residues of D-AA assisted tanning in the effluent would become the part of soil.

Table III. COD, BOD and TS analysis of unnatural D-AA tanned leather

Process	pH	COD (ppm)	BOD (ppm)	TS (ppm)	DS (ppm)	SS (ppm)
BCS	2.5-3	1000-2500	400-1000	30,000-60,000	29,000-57,500	1000-2500
Veg	4.0-5.0	1200-3000	500-1200	35,000-65,000	30,000-59,500	1300-2800
Ald	3.0-4.0	1200-3000	400-1000	20,000-40,000	25,000-48,500	800-1800
D-Ala+Ald	3.0-4.0	1200-3100	500-7000	16,000-37,000	24,000-45,000	500-1500
D-Glu+Ald	2.5-3.5	1100-3200	600-9000	18,000-38,000	22,000-42,500	700-1600
D-Lys+Ald	3.0-4.0	1300-3300	300-700	15,000-32,000	20,000-40,000	400-1400

It is known that the pickling process increases the TS of the effluent. In D-AA+Ald tanning the pollution of neutral salt is significantly decreased. In comparison with BCS tanning, the BOD5/COD value is greater for D-AA tanning than for BCS tanning, which demonstrates that the effluent of D-AA assisted tanning process is easier biodegradable than that of BCS tanning process.

4. Discussion

Unnatural D-AA are non-genetically-coded amino acids that either occur naturally or are chemically synthesized and becoming very important tools for chiral building block, conformational constraints, molecular scaffolds and also modern drug discovery and represent a nearly infinite array of diverse structural elements for the development of new leads in peptidic and non-peptidic compounds. Though natural and synthetic peptides formed of L-AA dominate applications in a wide variety of fields, it is largely felt that sooner the trend will favor peptides formed of D-AA [16]. The introduction of D-AA can significantly increase stability and resistance to proteolytic activity. Non-canonical AA in protein polymer design has been reviewed for development of biosensors, novel surfaces and materials [17]. To improve the structural consequences of D-AA in collagen triple-helical peptides have studied. And also recent computational study suggested that replacement amino acids residues L to D-conformation would stabilize the collagen triple helix [18].

In the study, skin/hide was tanned by ALD in the presence of three kinds of unnatural D-AA. The D-AA, i.e. D-Asp/Glu, D-Ala and D-Lys, that contain 1/2, 1/1 and 2/1 of NH_2/COOH groups respectively, were selected as bridging agents to enhance the stability to leather. As expected, enhanced stability and resistance to collagenases activity for the leather can be surely gained, when the cross-linking is conducted on the presence of basic D-Lys. However, surprising results were got in the presence of D-Ala or D-Asp/Glu. The former has less efficiency to improve the stability of the leather, and the latter even results in a worse stability. In addition, D-AA can be better than L-AA as glycine surrogates, retaining protein function without perturbing protein structure studied [19,20]. The possible reason is that the addition of D-AA changed the ratio of NH_2/COOH in the crosslinking. There are two competing reactions in the cross-linking system: the bridging linking between leather collagen molecules under the assistance of the added D-AA, and the blocking of the functional groups of the collagen matrix by the D-AA reagents. The ratio of collagen NH_2/COOH will determine which kind of reaction is dominant.

In general, the ratio of NH_2/COOH is about 2:3 in the goat skin. This is understandable that over the existence of one kind of group shall decrease the general cross-linking efficiency, leading to poor stability. For example, addition of Asp/Glu shall introduce more COOH groups than NH_2 groups, yielding far excess of COOH groups. One Glu molecule can just react with one amino group, so that the Glu not only loses the function of cross-linking bridge but also blocks a large amount of functional groups in the collagen molecules. As a result, the cross-linking efficiency significantly is decreased. In contrast to D-Glu, the addition of D-Lys can amend this unbalance of NH_2/COOH, resulting in

higher tanning efficiency. A green method for the self-aldol condensation of Ald using Lys studied [21]. This can explain that D-Lys can enhance the stability and resistance to collagenases activity in a wide range of concentrations. D-Lys+ald might also be involved in the process of polymerization of collagen networks. The permanent, D-AA ald crosslinking are associated with tough tanned leather that bear high leads and are subjected to minimal turnover and improve the mechanical strength of a particular quality, for example preventing lateral slippage between sub-elements of fibrils or offer a mechanism for interfibrillar bonding. D-AA along a peptide chain and protein fragments may be less digestible than the L-forms. D-D, D-L and L-D form of peptide bonds partly or fully inaccessible to proteolytic activities has been studied.

Furthermore, the optically active D-AA, accompanied by the improvement of the mechanical, thermal and biological stability of leather can be expected. AFM and SEM were used for revealing the influence of D-AA+Ald treatment on the microstructure. The results indicate that the leather could preserve the structure during the cross-linking treatment in the presence of D-AA. And also D-Lys improves the hydrophobicity of the leather collagen molecules. The introduction of D-Lys into collagen represents important advances in the chemistry of protein-based biomaterials. The hydrophobicity of peptides containing D-AA has been studied [22]. This D-AA assisted tanned leather demonstrates a higher resistance to microbial activities than conventional method. Recently we studied preparation and characterization of collagen matrix by unnatural amino acids as novel cross-linking bridges [23]. These D-AA proteins and peptides are longer-acting anti-microbial, antioxidant, anti-mutagenic and anti-carcinogenic activity than their L-enantiomeric analogues. Because D-Ala, D-Ser, D-Glu/Asp are an essential building blocks for peptidoglycan in bacterial cell walls. These unnatural D-AA, not usually found in natural proteins, have conformational attributes that are useful for the imposition of conformational stability and as structural probes.

4. Conclusions

Implementation of green chemistry to cleaner production and pollution prevention measures can provide both economic and environmental benefits. A green chemistry or eco-friendly approach to chrome-free tanning based on unnatural D-AA assisted tanning process has been developed. The properties of the leather are improved for example with regard to texture, thermal and mechanical strength and resistance to microbial activity. A further advantage is that the tanning wastewater is completely nontoxic and presents no disposal problems, either with regard to liquid waste or with regard to solid waste. In general, cleaner chrome-free tanning practice involves implementation of the following strategies: 1) able to shift from chemical to greener chemistry; 2) improvement of conventional tanning processes which helps to reduce the TS and other toxic chemical outputs; 3) use of greener chemistry systems where the uptake of the tanning agent is greater and improve the structural hydrophobicity; 4) improve interior interaction between collagen molecules, leading to an increase the mechanical and thermal stability; 5) increase the stability of triple helical structure of collagen via intra and inter molecular crosslink; 6) modify potential sites for physical, chemical and biological degradation; 7) significant reduction in tannery waste of pollution loads, toxic chemicals and controls the environmental problems. These D-AA assisted tanning technologies are regarded as eco-friendly and hence contribute to the development of cleaner process.

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