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Fibre stabilization before Enzyme-only beam house operation – An innovative approach for fail-safe chemical-free process

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Conventional beam house operations based on use of lime and sulphide followed in the global tanning industry are coming under the close scrutiny of the environmentalists on account of the possible adverse effect on human health and environment. Sulphide is toxic and the sulphide based dehairing operations also contribute to high COD load in the effluent due to the destruction of the hair. Lime which is used to the tune of about 10% on the weight of the raw hides and skins contribute to huge pile of sludge, the disposal of which becomes a problem. Enzyme-only beam house processes are being advocated to overcome the problems associated with the conventional process. A combination of protease and a lipase is recommended for achieving dehairing and defleshing and use of amylase or xylanase being screened for getting satisfactory fibre opening. However, enzyme based methods need a good control over process parameters to get satisfactory results in leather processing and to avoid damage to the pelt and the final leather. The enzyme treatment is most effective around physiological temperature and pH conditions and the hides and skins are susceptible to bacterial damage if the process is not accomplished in a very short time. Moreover, three classes of enzymes, most of the times, need to be used one after the other to achieve the best efficacy resulting in long process duration. This makes the material more prone to damage due to bacterial attack which may be made more acute due to the synergistic effect of the enzymes. To overcome all these problems associated with the enzyme-only process, an attempt has been made in the present investigation to stabilize the skin matrix with a tanning agent with the least negative influence on the activity of the enzymes so that the process duration can be extended for getting better efficacy without the risk of damage. Various tanning agents have been initially screened for their effect on the activities of the three chosen enzymes, protease (for dehairing), lipase (defleshing) and xylanase (fibre opening) and the tanning agent based on gluteraldehyde has been found to have the least effect on the activity of the enzymes. This tanning agent has been used for the fibre stabilization prior to enzyme-only process and its effect on the dehairing, defleshing and fibre opening has been studied and based on the study, a process has been standardized. The effect of such an approach on the final leather quality has also been studied and the results of the entire study would be presented in the paper.
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Introduction

The conventional liming and reliming processes for the removal of hair and other unnecessary proteins contribute high amount of COD, BOD, TDS etc., to the effluent[1-4]. The extensive use of sulphide in leather processing bears unfavorable consequences on the environment and the efficacy of effluent treatment plants[5]. Besides, sulphide is highly toxic with obnoxious odour causes serious health problems to the workers. To overcome the environmental damage and health related problems posed due to the use of toxic sulphide in unhairing, several researchers have tried and rationalized the enzymatic dehairing [6-12]. An important enzyme used in unhairing process belongs to the group of proteolytic enzymes, proteases. The main advantages of the use of proteases are specificity, activity under mild conditions, nonpollutnts

and biodegradability. But due to several reasons, commercial application of these enzymes for unhairing is not fully utilized by the global leather industry and still most of the tanneries are following hair-burning process, which destroy the hair completely. However enzyme-assisted lime-sulphide dehairing and fully enzyme based unhairing is being followed in some parts of the world.

Most of the enzymes and enzymatic methods developed in the past are applicable only for unhairing of hides/ skins in leather processing. The unhaired skins require fibre opening and this is traditionally done with the use of lime. Formation of large amount of lime sludge is the main drawback of lime. The treatment of lime in the form of calcium hydroxide removes all the interfibrous materials especially proteoglycans. Removal of glycosaminoglycans and particularly proteoglycans is necessary to allow the fibre structure to split apart. Otherwise, the fibre structure may be cemented together during drying. Hence in principle, fibre opening is the process that removes the proteoglycans and glycosaminoglycans from the skins and it is possible to produce pelt by removing the proteoglycans by using enzymes or by chemical auxiliaries. Few researchers have reported about the application of enzymes in fibre opening[13]. Xylanase enzyme has been used to acting upon the linear carbohydrate polymer thereby open up the rigid fibre structure of stingray skin[14]. Sodium laurul sulphate has been reported for the fibre opening of stingray skins[15] and it has the ability to bind with glycoproteins and proteins[16.] Even then, tanners are doubtful to use these enzymes to produce commercial leather because of certain disadvantages in using them. Enzyme based unhairing and fibre opening processes needs to follow a strict physiological condition. For example the soaked skins should be immediately treated with enzymes and it should not be aged for a prolonged period of time or else leads to putrefaction. The important factor in choosing enzymes as a dehairng and fibre opening agent depends on the specificity of the enzyme used and it should not attack the collagenous matter. But in enzymatic unhairing and fibre opening method it is necessary to treat the skins with enzymes for several hours and these enzymes may damage the collagenous structure. To overcome the limitations associated with the enzymatic dehairing and fibre opening process, in this investigation, the soaked skins were initially treated with a mild stabilizing agent and later with unhairing and fibre opening enzymes to achieve the optimum performance of these enzymes.

Materials and Methods

Protease, Lipase and Xylanase enzymes were purchased from Tex Biosciences (P) Ltd, earlier called Textan Chemicals (P) Ltd), Chennai and other chemicals were purchased from standard dealers in Chennai.

Improved Enzymatic Dehairing and Fibre Opening Method

Goat skins were used to test the efficacy of newly developed enzymatic dehairing and fibre opening process. Three sets of trials were carried out. In each set six wet salted goat skins were taken and marked as 1L 1R, 2L 2R, 3L 3R, 4R 4L, 5,6. The wet salted goat skins marked 1L, 2R, 3L, 4R and 5 were processed by a new unhairing and fibre opening process and it is given below.

***Raw Material*:** Wet salted goat skins

Soaking:

The skins were soaked for 6 h with 300% water and then washed with 2 changes of fresh water. The soaked weight was noted.

***Fiber Stabilization*: (percentage based on soaked weight)**

1.0-2.0 % Glutaraldehyde based tanning agent
Water 100%
Run the drum for 90' (4-5 rpm). Then the stabilized skins were taken for enzyme treatment.

Protease Treatment:
Water 100%
Protease enzyme 2%. Initially the drum was run for 30'. Then for every 1 h 5' for 48h (4-5 rpm). Then the skins were subjected to manual scudding.

Xylanase Treatment:
Water 100%
Xylanase enzyme 2%. Run the drum for 120' (4-5 rpm).

Lipase Treatment:
Water 100%
Lipasee enzyme 2%. Initially the drum was run for 30'. Then for every 1 h 5' for 8h (4-5 rpm). Then the skins were fleashed and scudded.

Pickling:
Water 80%
Salt 8% Run for 10 min.
Sulphuric Acid 1%
Water 20%
Given in 4 feeds at 10 min. interval and finally run for 60 min. pH of the pelt was kept at 2.8. Leave over night. Next day chrome tanning.

Chrome Tanning:
50% pickle water
Basic Chromium Sulphate 4% 30'
Basic Chromium Sulphate 4% 40'
Water 50% 30 min
Sodium formate 1% (in 10% water) 30 min
Sodium bicarbonate 1% 3x10min + 60min (in 10% water)
pH 3.8. Drain, rinsed and piled overnight. Next day, the tanned leathers were shaved and taken for further processing.

Effect of Glutaraldehyde on Protease Activity
The protease activity was determined by using the standard procedure. About 2ml of protease enzyme was added to the Casein substrate buffer solution and it was incubated for 30 min. at room temperature with the addition of different concentrations of glutaraldehyde. The reaction was stopped by the addition of 2ml of 5% TCA (tricloro acitic acid) and allowed to precipitate. Then the precipitate was centrifuged at 10,000rpm for 20 min. 1 ml of supernatant was drawn from each test tube. 2 ml of sodium hydroxide was added to each test tube. The contents are mixed well. Then 0.5 ml of Folin and Cicocalteau reagent was added and incubated at room temperature for 20 min. The observance was measured at 620 nanometer after the development of blue colour using UV visible spectrophotometer. The control was also performed by adding the enzyme after the inactivation of the substrate by the addition of TCA. The Tyrosine released by the enzyme was calculated from Tyrosine standard graph. The micromole of tyrosin released from the substrate is calculated from the graph.

Effect of Glutaraldehyde on Lipase Activity

The activity of lipase in the presence of g**lutaraldehyd**e was determined by using the standard procedure. About 75 ml of 2% poly vinyl Alcohol (PVA) was taken and mixed with 25.5 ml of olive oil (3:1) ratio. The emulsion obtained is homogenized for 10 min. 2.5 ml of emulsion is mixed with 2 ml of 0.05 molar phosphate buffer. After 10 min. 0.5 ml of enzyme sample was added with the addition of different concentrations of glutaraldehyde. The reaction was allowed to proceed for 20 min. Then 10 ml of acetone was added and the reaction mixture was titrated against 0.05 N NaOH.

$$\text{Lipase Activity} = \frac{(\text{Test} - \text{Control}) \times 1000 \times \text{N NaOH}}{\text{Incubation time}}$$

Effect of Glutaraldehyde on Xyalanase Activity

The activity of xylanase in the presence of glutaraldehyde was determined using the standard procedure using xylan as the substrate and the activity was determined in terms of the xylose liberated due to enzyme action. The xylanase activity was determined by the equation,

$$\text{Units /ml enzyme} = \frac{(\mu\text{mole of xylose liberated})\ (\text{dilution factor})}{\text{Time of assay (10 minutes) (0.1volume of enzyme used).}}$$

Conventional Beam house procedure

Wet salted goat skins marked as 1R, 2L , 3R, 4L, 6 were processed by conventional procedure. The raw skins were soaked with 300% water for 4 h and washed. Liming was carried out on soaked weight with 10% calcium hydroxide in the form of paste by adding 20% water. The paste was applied on flesh side of the skin and left over night. Next day the skins were unhaired and relimed with 10% calcium hydroxide and 150% water for 3 days in a pit. The defleshed, washed pelts were delimed with 1% ammonium chloride (% based on pelt weight) for 45 min in a drum to bring the pH 7.8 and washed. The delimed skins were treated with 8% salt and 80% water for 10 min and 1% sulfuric acid was added in 4 feeds at 10 min interval and finally the drum was run for 60 min to bring the pH 2.8. Then the pickled skins were processed using 8% Basic Chromium Sulphate (BCS) and 50% pickle water for 90 min. Then 50% water was added and the drum was run for 30 min. To the running drum 1% sodium formate (mixed with 10% water) was added. After 30 min 1% sodium bicarbonate (mixed with 10% water) was added in 3 feeds at 10 min interval and finally run for 60 min to bring the pH 3.8.

Post Tanning Procedure

The post tanning process is similar for both enzyme treated and conventional leathers and it is given below.

Raw material: Shaved wet blue goat leathers at 1.0mm thickness
Washing
Water 250%
Run for 10 minutes, drain.
Neutralization
Neutralizing syntan 1.0%
Run for 20 min.
Sodium formate 0.5%
Sodium bicarbonate 0.5%
Given in 3 feeds at 10 min. interval, finally run for 20 min., pH should be 5.0. Then washed twice with 200% water for10 min.

Retanning, Dyeing and Fatliquoring
Water100%
Resin syntan 3%
Run for 20 min.
Acid Dye 2% 30 min.
Synthetic fatliquor 4%
Run for 30 min.
Melamine based syntan 8%
Naphthalene based retanning syntan 8%
Run for 40 min.
Fatliquor based on sulfochlorinated hydrocarbons 4%
Polymeric fatliquor 3%
Fatliquor based on synthetic and neutral oils 4 %
Run for 40 min.
Fixing
Formic acid 2%
Water 20%
Given in 3 feeds at 10 min. intervals and finally run for 30 min.
Then the leathers were piled overnight. Next day, they were set, conditioned, again set with reversible setting machine and hooked for drying. After drying, the leathers were staked and buffed using 400 grit emery paper.

Histological studies with Hemotoxylin and Eosine Staining

The histological studies for the enzyme treated and conventional limed skins were carried out. After the completion of the above mentioned processes, the samples were cut and preserved in 10% formalin for 48 h. The fixed samples were dehydrated in a series of solutions of alcohol of different concentrations (50 to 100%) and then cleared in xylene. They were finally embedded in paraffin wax into moulds. The moulds were labeled and stored until use. Thin sections (10µm thick) were cut on a microtome, mounted on glass slides and stained with hematoxylin and again counterstained with eosine.

Scanning Electron Microscopic Analysis

In order to study the effect of the newly developed unhairng and fibre opening method on the structural characteristics of the leathers produced, scanning electron microphotographs of control and HMH-chromium composite treated leathers were compared. The samples measuring 5mm x 2mm were cut from the crust leathers using fresh stainless steel blades. The samples were mounted both vertically and horizontally on aluminum stubs using an adhesive. These were then coated with gold using an Edwards E-306 sputter coater. The stubs were introduced into the specimen chamber of a FEI-Quanta 200 scanning electron microscope. The stubs mounted on the stage could be tilted, rotated and moved to the desired position and orientation. The micrographs for the cross-section were obtained by operating the microscope at higher voltage.

Physical Testing and Visual Assessment

The samples for physical testing were cut from the enzyme treated and conventional goat crust leathers from the official sampling position[17]. The samples were conditioned to the required relative humidity of 65±2% at 20±2°C for 48 h as per IUP 3[18]. The tensile and tear strengths were measured as per the standard procedures[19,20]. Double edged tearing method which is also called as Baumann tear was used to measure the tear strength. Experienced leather technologists assessed the organoleptic properties such as

general appearance, grain smoothness feel and area yield. The leathers were rated on a scale of 0-10 points for each functional property, where higher points indicate better property.

Results and Discussion

Effect of Fibre Opening Enzyme on Goat Skin

Xylanase enzyme has been effectively used to acting upon this linear carbohydrate polymer thereby open up the rigid fibre structure. The enzyme xylanase plays an important role in the opening up of fibre bundles of goat skin thereby able to remove the proteoglycans. Removal of glycosaminoglycans and particularly proteoglycans is necessary to allow the fibre structure to split apart. Otherwise, the fibre structure may be cemented together during drying. The optimum amount of enzymes employed in this process has precise action on goat skin and it is obvious that it would not contribute much pollution load to the effluent.

Effect of Glutaraldehyde on Protease Activity

The protease activity has been determined in the presence of different concentrations of glutaraldehyde and is presented in Table 1. The table shows the result for protease (1ml) and the activitiy(1.7115)µg/ml/min. In the presence of glutaraldehyde, the protease activity is increased and is shown below.

Table 1. Effect of Glutaraldehyde on Protease Activity

Protease enzyme(ml)	GT 50	Enzyme activity(µg/ml/min)
1 ml	-	1.7115
1 ml	200µl	4.071
1 ml	400 µl	4.137
1 ml	600 µl	4.14
1 ml	800 µl	4.14
1 ml	1000 µl	4.14

Effect of Glutaraldehyde on Lipase Activity

The lipase activity has been determined in the presence of different concentrations of glutaraldehyde and is presented in Table 2. The table shows the result for Lipase (0.5ml) and the activity (0.5) U/ml. In the presence of glutaraldehyde, the lipase activity is decreased, but the activity does not change with increasing concentration of glutaraldehyde.

Table 2. Effect of Glutaraldehyde on Lipase Activity

Lipase enzyme(ml)	Glutaraldehyde	Enzyme activity(U/ml)
0.5 ml	-	0.5
0.5 ml	200µl	0
0.5 ml	400 µl	0.25
0.5 ml	600 µl	0.25
0.5 ml	800 µl	0.5
0.5 ml	1000 µl	0.5

Effect of Glutaraldehyde on Xylanase Activity

The xylanase activity has been determined in the presence of different concentrations of glutaraldehyde and is presented in Table 3. In the presence of glutaraldehyde, activity increases with the increasing concentration of gluteraldehyde upto a concentration level of 800 µl/ml. This gives an indication that gluteraldehyde may not affect the xylanase activity rather might help in increasing the efficacy of the enzyme which will be beneficial with the substrate stabilized with it.

Table 3. Effect of Glutaraldehyde on Xylanase Activity

S. No	Xylanase Enzyme (ml)	GT 50	Activity(U/ml)
1.	0.1 ml		33
2.	0.1 ml	200µl	33
3.	0.1 ml	400 µl	40
4.	0.1 ml	600 µl	47
5.	0.1 ml	800 µl	53
6.	0.1 ml	1000 µl	53

Effect of Enzymes on Fibre Opening: Histological Studies with H & E Staining

The optical microphotographs taken for the enzyme treated and limed goat skins are set out in Figures 1 and 4. Glycosaminoglycans are strongly anionic, unbranched long-chain polysaccharides containing aminated monosaccharide. The complex molecules formed by the attachment of glycosoaminoglycan chains to a protein core constitute the proteoglycans. Some of the significant constituents of connective tissue matrices of skin are proteoglycans. Removal of the non-collagenous proteins is necessary to produce soft leather. The microphotographs reveal that xyalanase treatment opens up the fibre bundles of goat skin. The optimum amount of enzymes added during fibre opening splits the fibre structure and makes the skin opened up. This opening up allows penetration of tanning and lubricating agents to produce leather with range of organoleptic properties required by the leather manufacturers and consumers.

Effect of Enzymes on Fibre Opening: SEM Studies

Scanning electron microphotograph of enzyme and lime treated goat skins showing their surface at a magnification of 100x are presented in Figures 5 and 6. SEM pictures showing their cross sections at magnifications 500x and 100x are presented in figures 7-10. The microphotographs confirm that the enzymes added for fibre opening have the ability to open up the skin structure which is clearly presented in SEM Figures.

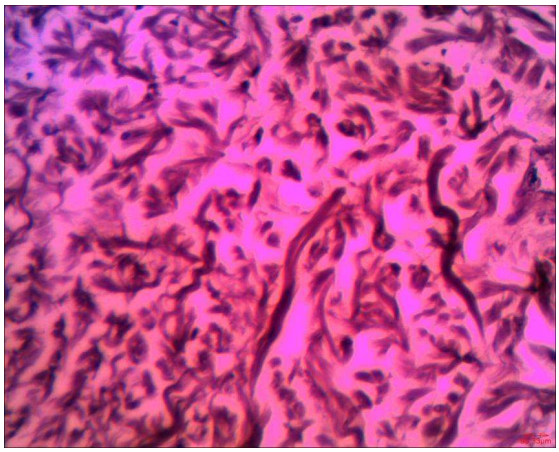

Figure 1. Optical microphotograph of Enzyme Treated goat skin 100X

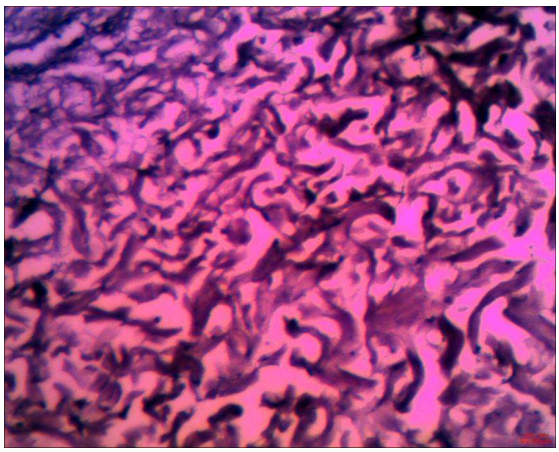

Figure 2. Optical microphotograph of Lime Treated goat skin 100X

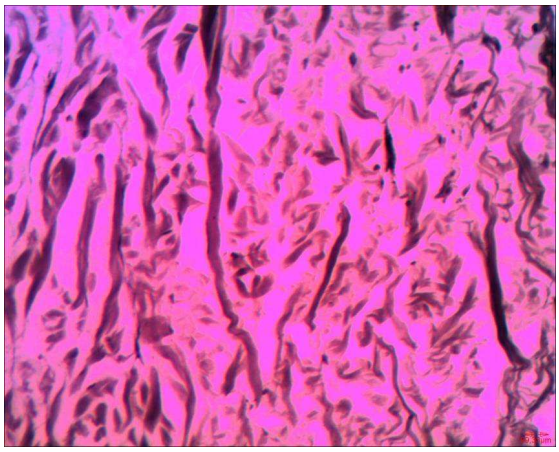

Figure 3. Optical microphotograph of Enzyme Treated goat skin 200X

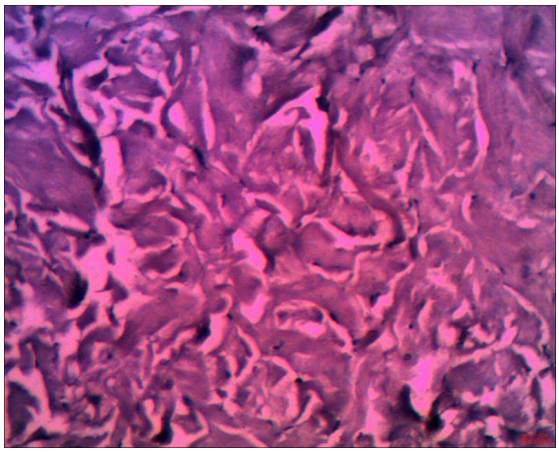

Figure 4. Optical microphotograph of Lime Treated goat skin 200X

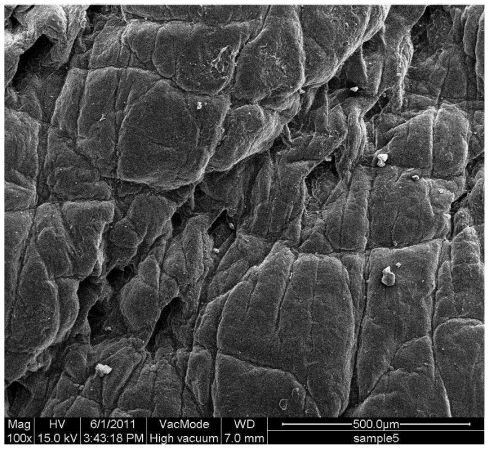

Figure 5. SEM picture of Enzyme Treated goat skin 100X

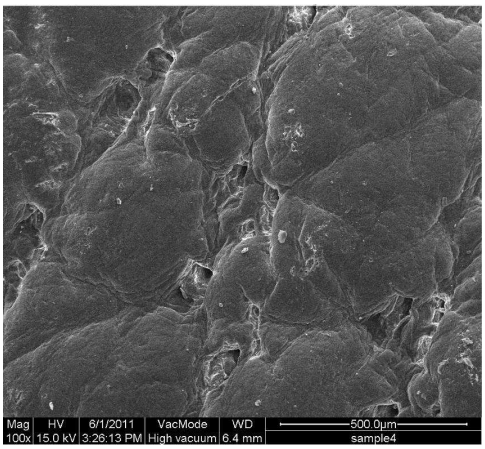

Figure 6. SEM picture of Lime Treated goat skin 100X

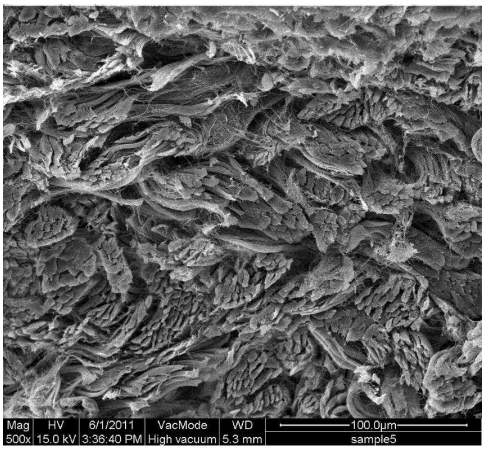

Figure 7. SEM picture of Enzyme Treated goat skin 500X (cross section)

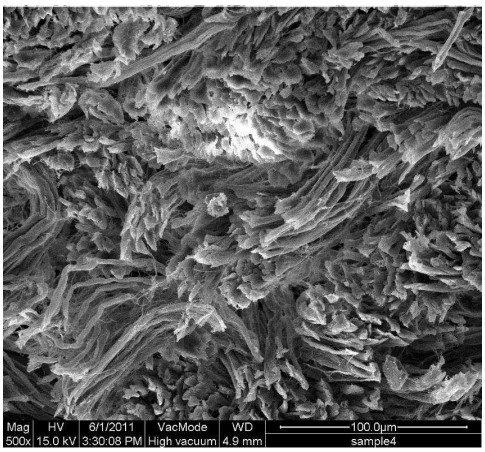

Figure 8. SEM picture of Lime Treated goat skin 500X (cross section)

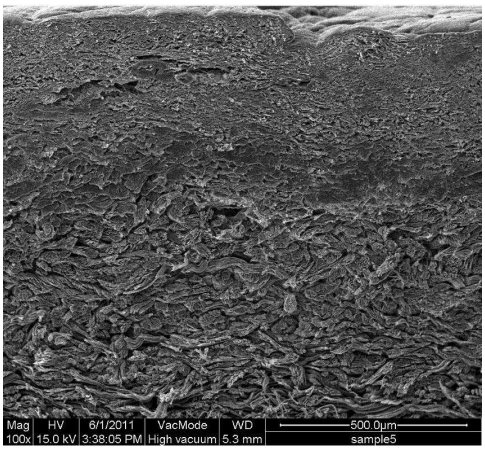

Figure 9. SEM picture of Enzyme Treated goat skin 100X (cross section)

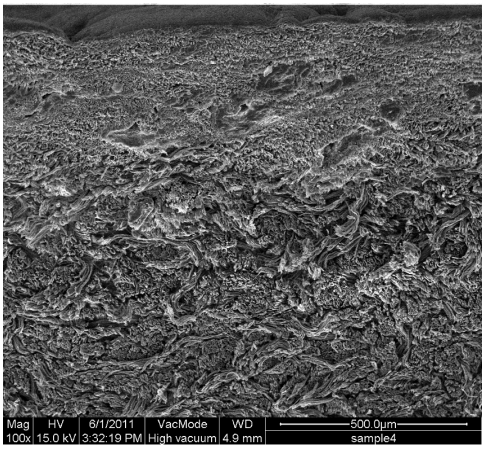

Figure 10. SEM picture of Lime Treated goat skin 100X (cross section)

Physical Testing and Visual Assessment Data

The strength and organoleptic properties of enzyme treated and control crust leathers are given in Tables 4 and 5. From the table, it is clear that the strength properties of enzyme treated crust leathers are similar to that of conventional leathers. The grain smoothness property is better for enzyme treated leathers and also found to have improved area yield.

Table 4 Strength properties of crust leathers

Strength Properties	Enzyme Treated	Conventional
Tensile Strength kg/cm^2	245 ± 6	256±3
Elongation at break %	88±2	84±1
Tear Strength kg/cm	96±4	98±3
Grain crack load, kg	26±1	28±2
Distension at grain crack in mm	9.2±0.1	9.3±0.2

Table 5 Visual Assessment Data* (scale 1-10)

Properties	Enzyme Treated	Conventional
General Appearance	8 ± 1	8 ± 1
Grain smoothness	9 ±0.5	8±1
Feel	8 ± 1	7 ± 1
Area yield	8 ± 1	7 ± 1

*** Assessment by experienced leather technologists**

Conclusions

A chemical-free enzyme-only beam house operation based on protease, xylanase and lipase has been attempted to eliminate the use of lime and sulfide in leather processing. The problems encountered with prolonged enzymatic process resulting in damage to the substrate could be avoided by the innovative approach made in the present study, i.e., by stabilizing the skins with glutaraldehyde before enzyme treatment. This enzyme-only beam house process afforded leathers that are comparable to those made by conventional liming process.

References

1. Thanikaivelan P, Rao J R, Nair BU & Ramasami T, Zero discharge tanning: A shift from chemical to biocatalytic leather processing, *Environ Sci & Technol*, **36** (2002) 4187-4194.
2. Ramasami T & Prasad B G S, Environmental aspects of leather processing, Proc LEXPO XV, (ILTA, Calcutta) 1991, 43-71.
3. Marsal A, Cot J, Boza E G, Celma P J & Manich A M, Oxidizing unhairing process with hair recovery. Part I. Experiments on the prior hair immunization, *J Soc Leath Technol Chem*, **83** (1999) 310–315
4. Taylor M M, Bailey D G & Feairheller S H, A review of the uses of enzymes in tannery, *J Am Leath Chem Assoc,* **82** (1987) 153-163.
5. Bailey D.G., Tunick M.H. and Kuzma M.A. (1982), 'Effect of sulfide, chromium and phosphate ions on methane production by an anaerobic sludge acclimatized of tannery beamhouse effluent', Proceedings of the 14th Mid Atlantic Industrial Waste Conference, June 27-29, College Park, Maryland.
6. Rao M B, Tanksale A M, Ghatge M S & Deshpande V V, Molecular and biotechnological aspects of microbial proteases, *Microbiol & Mol Biol Rev*, **62** (1998) 597-635.
7. Foroughi F, Keshavarz T & Evans C S, Specifities of proteases for use in leather manufacture, *J Chem Technol Biotechnol*, **81** (2006) 257-261.
8. Gehring A G, Dimaio G L, Marmer W N & Mazenko C E, Unhairing with proteolytic enzymes derived from streptomyces griseus, *J Am Leath Chem Assoc,* **97** (2002) 406-412.
9. Paul R G, Mohamed L, Davighi D, Addy V L & Covington A D, The use of neutral protease in enzymatic unhairing, *J Am Leath Chem Assoc,* **96** (2001) 180-185.
10. Onifade A A, Al-Sane N A, Al-Musallam A A, & Al-Zarban S. A review: potentials for biotechnological applications of keratin degrading microorganisms and their enzymes for nutritional improvement of feathers and other keratins as livestock feed resources, *Biores Technol,* **66** (1998) 1-11.
11. Dayanandan, J. Kanagaraj, Lesley Sounderraj, R. Govindaraju and G. Suseela Rajkumar **(**2003) Application of an alkaline protease in leather processing: an ecofriendly approach. *Journal of Cleaner Production* **11**, P 533-536.
12. Sivasubramanian.S,Murali Manohar.B, Rajaram.A and Puvanakrishnan.R(2008) Ecofriendly lime and sulfide free enzymatic dehairing of skins and hides using a bacterial alkaline protease. *Chemosphere* Volume **70**, Issue 6, Pages 1015-1024.
13. Thanikaivelan P, Bharath C K, Saravanabhavan S, Rao J R, Chandrasekaran B Chandrababu N K & Nair B U. Integrated hair removal and fiber opening process using mixed enzymes *Clean Techn Environ Policy* (2007) **9**: 61–68.
14. Karthikeyan.R, Chandra Babu.N.K, Mandal.A.B and Sehgal.P.K 'Studies on the Preparation of Soft Leathers from Himantura (Family Dasyatidae) Stingray Skins and their Applications in Foot and Hand Reflexology' **XXX IULTCS** *Congress Proceedings, Beijing, China* during October 11-14, 2009.
15. Karthikeyan.R, Chandra Babu.N.K, Mandal.A.B and Sehgal.P.K 'A new depigmentation and fibre opening method for the conversion of stingray skins into leathers', *Journal of the American Leather Chemists Association* (2011), Vol**105**, 26-33.
16. Pitt-Rivers R. and Impiombato F.S. (1968), 'Binding of sodium dodecyl sulphate to various proteins', *Biochem. J.*, Vol.**109**, pp.825-830.
17. IUP: 2 Sampling, *JSLTC* **84**, 303, 2000.

18. SLP 3 (IUP 3), The Society of Leather Technologists and Chemists, Northampton, 1996.
19. IUP: 6 Measurement of tensile strength and percentage elongation, *JSLTC* **84**, 317, 2000.
20. IUP: 8 Measurement of tear load –Double edge tear, *JSLTC* **84**, 327, 2000.