Paper B42

Enzyme consortium of *Aspergillus tamarii* MTCC5152 for greener beam house processing of hides

Muthiah Shanmugavel[1], Arunachallam Premalatha[1], Anandan Dayanandan[1], Ramakrishnan Ramesh[3], Arumugam Gnanamani[1], Lesley Sounderraj, Narasiman Kannan Chandrababau[3], Alla Jayaprakash[3] , Asit Baran Mandal[2] and Gopal Suseela Rajakumar[1*]
Microbiology Division[1], Chemistry Division[2], Tannery Division[3] Central Leather Research Institute, Adyar, Chennai 600 020 India. Phone: 91-44-24451399; Fax: 91-44-24911589, *Email: suseela_rajkumar@yahoo.com

Abstract
The conventional beam house processes employ a wide range of chemicals leads to more than 60% of the total pollution in leather processing. The leather industry is undergoing a paradigm shift towards bioprocessing to reduce the pollution levels. Even though alkaline proteases have been available commercially for still proteases with wide spectrum of activity to dehair a whole range of hides and skins are looked for to combat pollution. The enzyme α-amylase acts on proteogycans and holds great potential for fiber opening.*A.tamarii* MTCC5152 produces an alkaline protease and α-amylase resistant to protease and both enzymes were produced by SSF in Koji room. The protease has been studied to have potential to dehair both hides and skins. These enzymes were studied for dehairing followed by fiber opening processes. The Indian cow hides were processed by pile method without the use of lime and sodium sulfide. Complete dehairing was observed after 18-24 h at room temperature (30- 32°C). The opening up of the fiber structures of the collagen fibers was very good. These enzymes in a consortium are the prospective enzyme preparation and can be used for processing cow and buffalo hides without the use of chemicals. The pollution parameters (BOD, COD, TSS and TDS) were reduced to 40- 60% and there is reasonable reduction in water consumption, wastewater discharge, when compared to the conventional leather processing.

Introduction
Liming and reliming has been particularly a problematic area as they contribute to 60% of the total pollution load in leather processing [1] The conventional dehairing processing with sodium sulfide and lime is responsible for 84% of BOD, 75% of the COD, 92% of suspended TDS[2] . Enzymatic process reduces the effluent, avoids the use of toxic chemicals, and simplifies the pretanning processes and complete recovery of hair/wool and easy handling of the pelts without any discomfort is possible. In view of the recent trend of developing ecofriendly technologies[3] the enzymes, protease and α-amylase produced by *A.tamarii* MTCC5152 have been used in this study as a consortium to evaluate their dehairing and fiber opening potential in beam house processing.

A.tamarii MTCC5152 produces both protease[4,5] and α-amylase in good yield in the same cultivation medium by solid state fermentation (SSF). The secretion of both these enzymes by this fungus in the same culture medium is advantageous for an integrated approach of performing both dehairing and fiber opening processes in a single or in two consecutive steps for beam house processing. In this work, the use of these enzymes as a consortium in dehairing by paste method, followed by a second treatment of the same enzyme consortium by drumming method for fiber opening of cow hides has been studied in comparison to the chemical method. Visual and microscopical examinations of the processed hides have been made to confirm the results. The performance of the crust leathers has been evaluated in terms of bulk properties and the results are discussed.

Materials and methods
Salted cow and buffalo hides were obtained from a commercial unit processing hides/ skins. The enzyme consortium is obtained from *A.tamarii* MTCC5152 isolated from soil. The enzyme

consortium prepared from the fungus produced 140 u/g protease activity and 75 u/g α- amylase activity. All other chemicals used were of commercial grade.

Production of protease and α-amylase by solid state fermentation by *A.tamarii* MTCC5152

The enzymes protease and α - amylase have been produced by solid-state fermentation (SSF) at pilot scale level using wheat bran as the substrate in Koji room based on conditions standardized at lab scale studies. SSF was conducted using 2 Kg capacity aluminium trays (20 nos). The trays containing the substrate were sterilized and the moisture content of the substrate was maintained at 70% moisture level. Fungal spores of *A.tamarii* MTCC5152 obtained from a fully fermented wheat bran substrate containing 1X 10^6 spores/ml were added as inoculum to each tray and mixed well and incubated at 26-28^{0}C for a period of 72-96 h at 90-95% humidity. Sampling was done at 24 h intervals to ascertain fungal growth and enzyme activity.

Enzyme extraction and ultra concentration

The fermented wheat bran substrate was mixed with Borate buffer (pH 9.5) in the ratio of 1:10 and soaked for 30 min at 4° C and kept in the shaker for another 30 min. After mixing the crude enzyme extract was filtered through nylon mesh, centrifuged at 10,000 rpm for 20 min at 4ºC. The supernatant was collected and stored at 4ºC and used as a crude enzyme. The crude enzyme was loaded in to the ultra concentrator and the nitrogen gas was applied to give the pressure. The ten fold concentrated enzyme was collected and precipitated using 80% w/v ammoniun sulphate. The precipitate was collected after centrifugation, lyophilized and an enzyme formulation was prepared using different enzyme stabilizers and stored at 4 ºC.

Assay for protease activity

Protease activity was determined according to the method of Anson[6] (1938) with slight modification using casein as the substrate followed by Berla Thangam & Suseela Rajakumar[7] (2000). The enzyme powder was dissolved in different concentration in 20 mM borate buffer (pH 9.0) containing 2 mM $CaCl_2$. The amount of protease activity was determined using a standard graph prepared from tyrosine. One unit of protease activity (U) was defined as the amount of enzyme required to produce 1 mg of tyrosine per ml of enzyme in 30 min at 55 °C.

Assay for α - amylase activity

The enzyme α- amylase activity was assayed by the Dinitrosalicylate (DNSA) method of Miller[8] (1959) using 0.3 % starch as the substrate. The - amylase activity was determined using a standard graph prepared from maltose. One unit of α - amylase activity (U) was defined as the amount of enzyme required to liberate 1 mg of maltose/ml/min at 55°C.

Dehairing process using protease from *A.tamarii*

Salted cow/buffalo hides (2ft X 2ft size) were soaked to remove the salt following conventional procedure, drained and used for bioprocessing studies. The enzyme powder was used to prepare an enzyme paste by mixing with borate buffer (20 mM) to adjust the enzyme paste to pH 9.0 (optimum pH for protease) and applied on the flesh side of the hides. The hides were folded flesh to flesh and piled at room temperature and assessed for their property after 18-24 h. Soak method was also studied by soaking the hides in various concentrations of enzyme powder using 20% v/w water.

Enzyme based fiber opening

An enzymatic pathway without lime forms a 'green' route for and fiber opening. using enzyme formulation without sodium sulfide has been designed for hides at an operational pH of 9.0. After the enzymatic, the hides were washed and different concentrations of α-amylase from *A.tamarii* MTCC5152 was added with 20% water and subjected to drumming for 4 h with intermittent drumming with breaks for 30 min for every 30 min run (fiber opening). The hides and the liquor were

checked for their pH levels, processed to crust leather and the leather quality was assessed by testing the strength properties. The hides after fiber opening process were also evaluated by microscopical analysis after staining.

Estimation of hexosamine from hides

The hexosamine content of cow hide pieces an indication of fiber opening of hides/skinswere determined by estimating the amount of pyrrole condensation product formed by the reaction between hexosamine and alkaline solution of acetyl acetone. The pyrrole that is formed gives a purple red colour with an alcoholic acid solution PDAB (Para- dimethyl amino benzaldehyde) which is read at 530 nm. About 0.2 g of hide samples were washed with physiological saline and cut into small pieces, defatted with chloroform: methanol (2:1v/v) and dried. 0.1 g of dried hide pieces were hydrolyzed with 5 ml of 6N HCl at 110 ^{o}C for 18-20 h in sealed glass tubes. After hydrolysis the samples were evaporated to dryness and the residue was dissolved in water and made up to a known volume. The hexosamine content was estimated by the method of Elson and Morgan[9] (1933).

Histological studies of processed hides

Hide pieces obtained after dehairing and fiber opening process were preserved in 10 % formalin for histological studies[10]. Formalin preserved hide samples were washed thoroughly with water and embedded in molten wax and wax blocks were made. The blocks were sectioned with microtome and placed on the slides. The sections were washed in tap water, rinsed in distilled water for 30 seconds and stained with hematoxylin for 2-3 min. The stained slides were washed in water and then dipped 8 times in a container of absolute ethanol. The slides were then dipped in primary container of xylene until the wax sheets slide off evenly. This procedure was repeated for 10 times in a secondary xylene container until the saturation is achieved. The slides were kept in a third container of xylene until ready to apply cover slip. Once the slides were ready, cover slips were fixed with DPX (mountant) on the slide. The dried slides were viewed through microscope (Nikon) at 4x100 X magnification.

Results and discussion

The crude extracellular enzyme extract obtained from *A.tamarii* MTCC5152 was assessed to have both protease and α-amylase activities and in good concentrations. To ascertain the suitability of these enzymes for leather processing, protease and α-amylase activities from *A.tamarii* MTCC5152 were estimated at different pH for its activity and stability. The crude protease has an optimal activity at pH 9.0 (Fig.1). The enzyme is active over a broad pH range of 8- 10. Similar studies were conducted with α-amylase for the purpose of fiber opening studies. The results show that α-amylase has maximal activity at pH 6.5 (Fig.2) and the enzyme showed good pH stability in the pH range of 5.0- 7.0 with moderate activity at pH 8.0-9.5. Presence of α-amylase in operation may thus helps in the splitting of collagen fiber bundles alongside enzymatic , providing an opportunity to avoid liming and deliming. The pH activity and stability relationship shows that α-amylase can be used in the fiber opening process without any change in the process operation.

Table.1 shows the effect of enzyme concentration on dehairing of cow and buffalo hides in both paste and soak method. In both the methods cow / buffalo hides were completely dehaired by applying 3% w/w of the enzyme powder. At 2%w/w concentration moderate dehairing was obtained. The dehaired hides were washed and the pH of the resultant hides was at pH 6.5. Fiber opening of cow hides was conducted using α-amylase obtained from *A.tamarii* MTCC5152. The hides after enzyme based beam house processing were assessed for hexosamine content. Data on hexosamine content of the hide samples after treatment with enzymes shows (Fig-3) that by using 0.5 - 1%w/w α-amylase maximum degradation of preteoglycan was removed from the cow hides. The results also show that hexosamine removal was better in α-amylase treated hides than that of protease alone and lime and sulphide treated hides (control) indicating the potential of this enzyme for beam house operation for bioprocessing. 1% w/w enzyme in drumming method could give results better than the control samples (Fig-3). The results show that the enzyme treated hides/leathers were comparable to control

samples processed with lime – sulfide, in certain properties better than the control leathers as evidenced from microscopical analysis of hides (Fig- 4) and from physical properties of tanned leather.

Fig-1. Effect of pH on protease activity

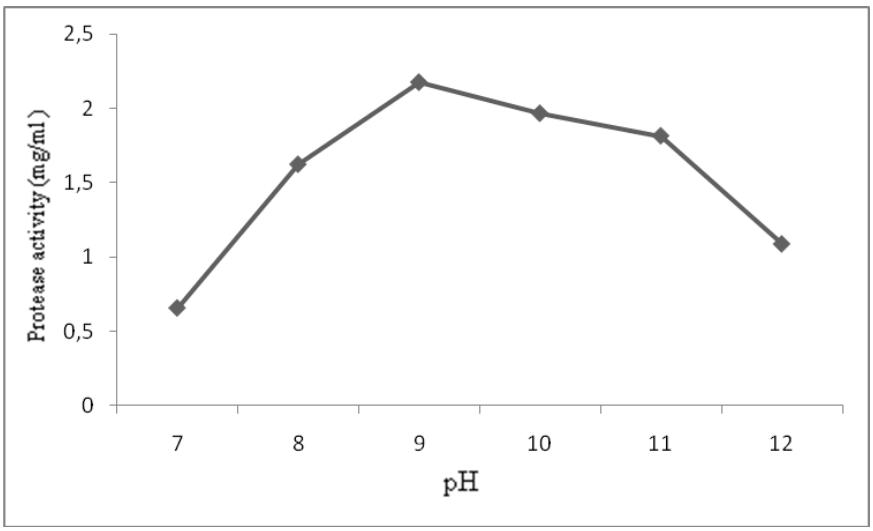
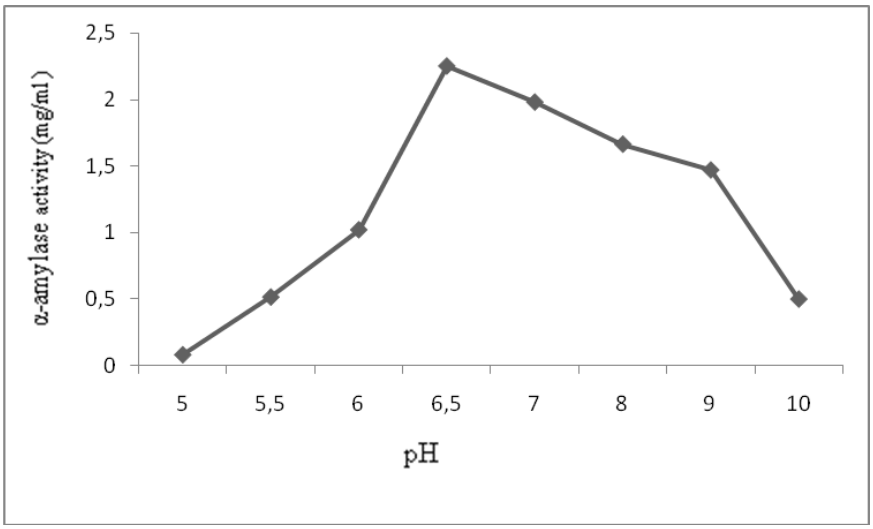


Fig-2. Effect of pH on α-amylase activity



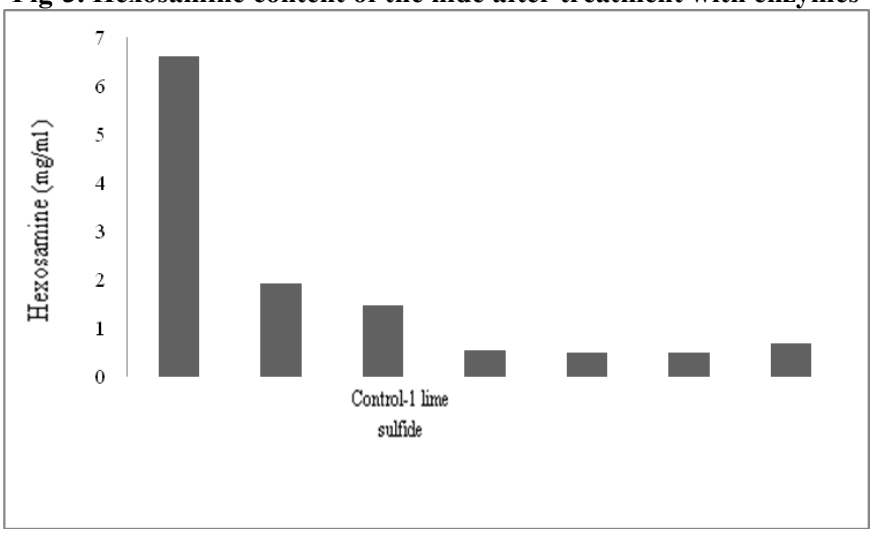
Microscopic analysis of hide samples after dehairing (3% w/w enzyme) and fiber opening process (1% w/w enzyme) using enzyme consortium containing protease and α- amylase from *A.tamarii* MTCC5152 is given in (Fig. 4.), respectively. It's clear from the results that the enzyme treated hides were dehaired without any trace of hair and the fiber opening was good comparable (Fig.4b) to the leather prepared by traditional methods (Fig.4a). This could be due to the actions of the presence of α-amylase in the enzyme preparation used for dehairing process. The enzyme acted very efficiently on the hide, removed the interfibrillary materials by degrading the carbohydrate moieties present in the glycoprotein through enzymatic action. Similarly, the presence of protease in the enzyme consortium used for fiber opening step helps in the removal of any root hair and scuds present in the hides during processing (Fig.4c and Fig.3).

Table-1. Effect of *A.tamarii* enzyme consortium for dehairing and fiber opening

XX- Slight dehairing; XXX –Moderate dehairing; XXXX-- complete dehairing; ---Not done; +++- Moderate fiber opening, ++++- Good fiber opening

Process details	Enzyme concentration (% w/w) dehairing and fiber opening index after 24 h				
	0.5%	1%	2%	3%	4%
Paste method (cow hide)	-	XX	XXX	XXXX	XXXX
Soak method (cow hide)	-	XX	XXX	XXXX	XXXX
Paste method (Buffalo hide)	-	XX	XXX	XXXX	XXXX
Soak method (Buffalo hide)	-	XX	XXX	XXXX	XXXX
Fiber opening (cow hide)	+++	++++	++++	++++	++++

Fig-3. Hexosamine content of the hide after treatment with enzymes



The subjective assessment of leathers by hand evaluation for both control and experimental leathers are presented in a 0-10 point scale. It is apparent that the experimental leathers exhibited comparable properties to that of control leathers (Fig. 5 and Fig. 6). Properties like softness, fullness, grain tightness are improved in enzyme treated leathers compared to control leathers. Thanikaivelan et al. (2006)[11] reported similar trends in integrated hair removal and fiber opening process using commercial mixed enzymes, protease and α-amylase.

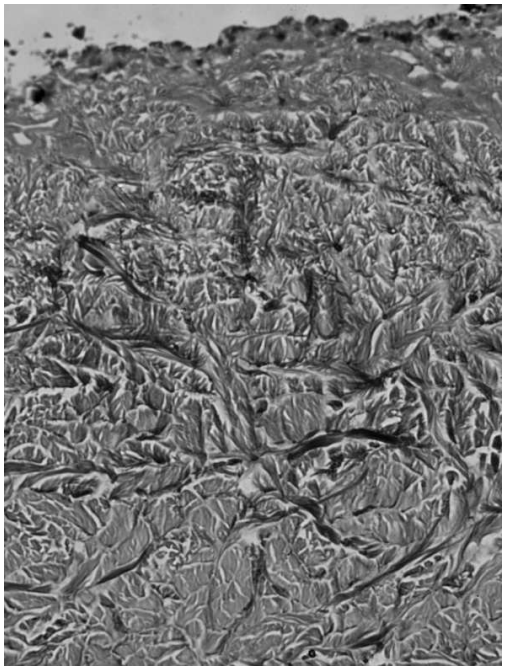


Fig- 4a. Photomicrograph of unhaired Cow hide (Control)

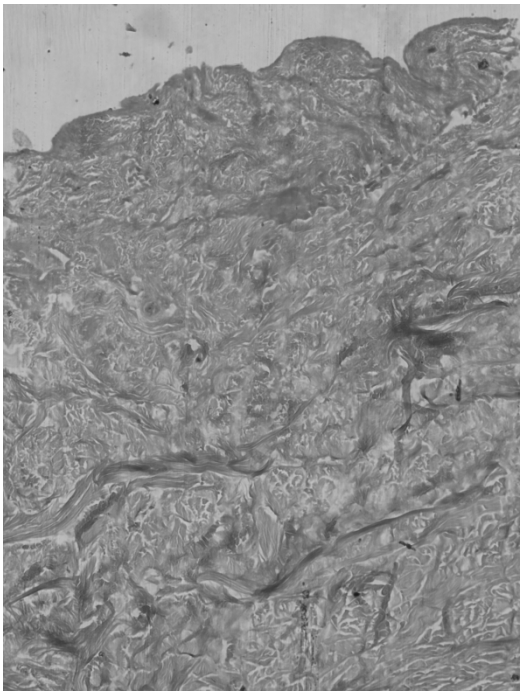


Fig- 4b. Photomicrograph of unhaired Cow hide (Enzyme treated 3%)

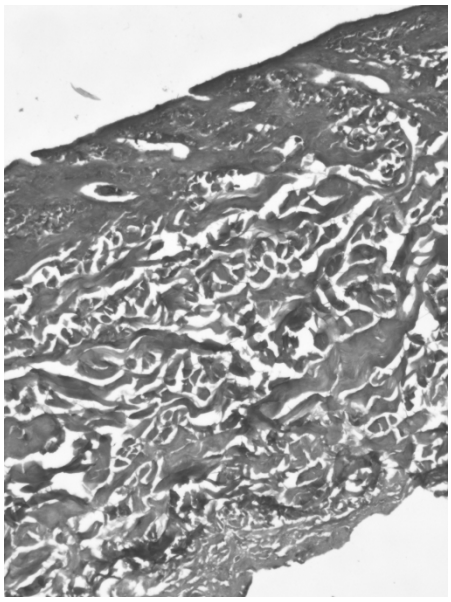


Fig- 4c. fiber opening (Control)

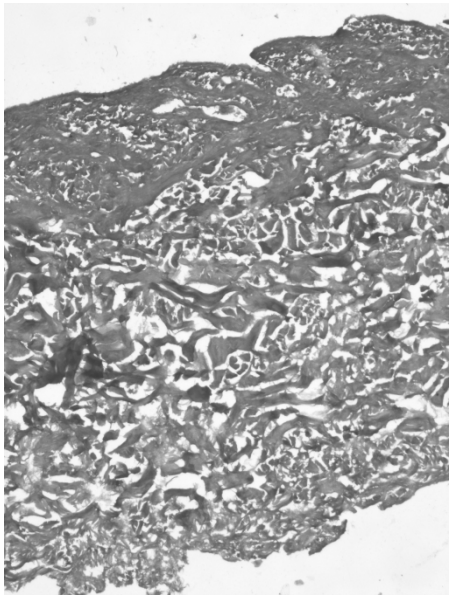


Fig- 4d. fiber opening (1% α- amylase)

Fig.5. Hand and visual of wet blue leather

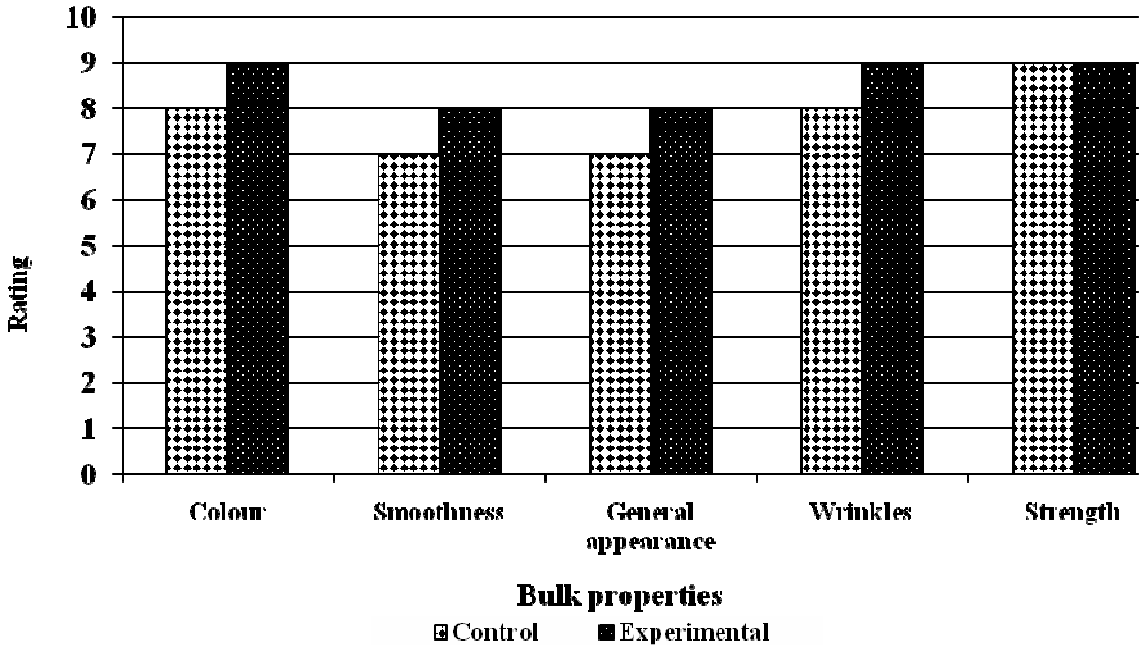
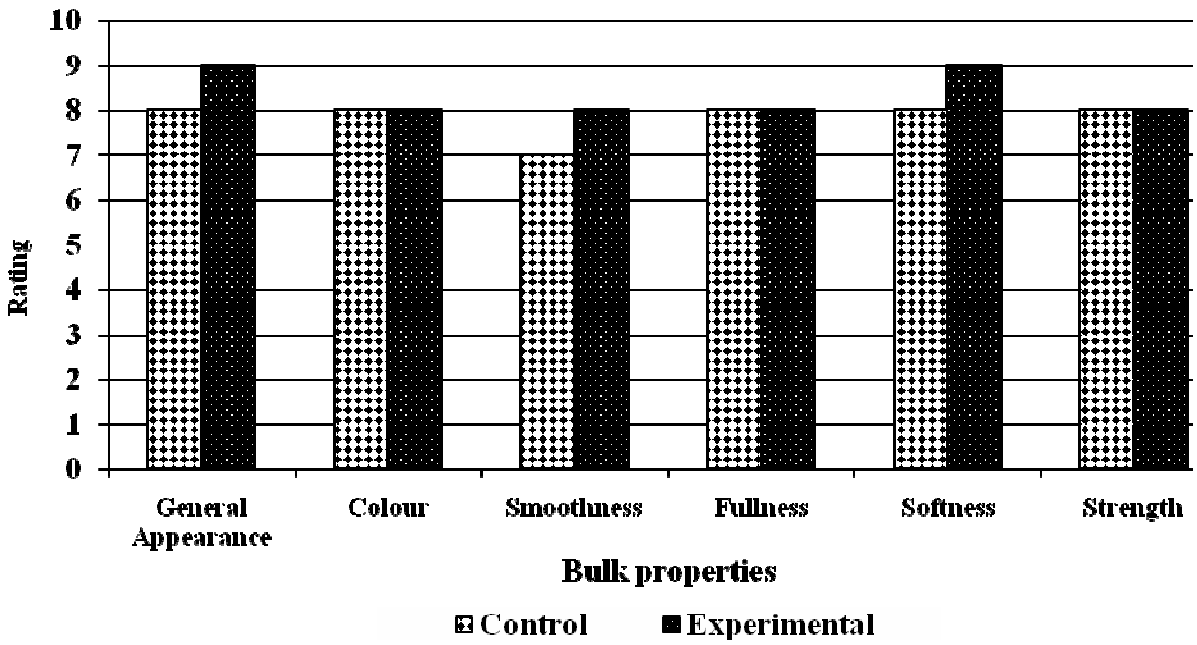


Fig.6. Hand and visual assessment of crust leather



Conclusions

Enzyme-based bioprocessing including dehairing and fiber opening is a paradigm shift away from conventional chemical-based leather processing. It can be seen from all the experimental results that the enzymatic and fiber opening of cow hides using protease and α- amylase from *A.tamarii* MTCC5152 as a consortium is an efficient two step process and for fiber opening option with a

potential for reduction of pollution considerably and the hair can also be saved without any degradation. Enzyme based and fiber opening also improves area yield and reduces wrinkle formation. In this work, the entire beam house processes have been modified to achieve clean and green leather processing through the use of enzymes for lime-free and fiber opening in beam house operation. This approach obtains a clean pelt with the desired fiber opening and elimination of lime sludge and considerable water and chemical reduction in the processing.

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