Paper B41

Enzymatic dehairing of goat skins utilizing protease from *Pseudomonas fluorescens* species isolated from fish visceral wastes

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Abstract

Leather is one of the major unique commodity that links the rural farmer to the fashion world. Leather as a natural material offers numerous advantages over synthetics, namely, aesthetic appeal, feel, texture and breathability. Though the leather industry has brighter sides of catering the high-tech fashion industry, it also has a darker side of polluting the environment. Biotechnology extends a warm hand by the utilization of enzyme in tanning industry which is likely to reduce pollution to zero level. The most constrained operation from environmental point of view is the dehairing of skin/hide.

An attempt has been made in this study, for the first time, to extract crude protease from *Pseudomonas fluorescen*s species from fish visceral wastes and utilize for dehairing of goat skins. The bacterial species isolated from the fish visceral wastes have been identified as *Pseudomonas fluorescen*s by biochemical tests and microscopic examination. The optimum pH and temperature conditions of the extracted crude protease for maximal activity were found to be 10.5 and 40˚C, respectively. It has been found that a combination of extracted crude protease (6%) with reduced amount of sodium sulphide (0.5%) provides complete dehairing of goat skins with complete elimination of lime. The higher usage of enzyme is owing to its crude nature and requires further purification for reducing the dosage. Hand, physical and scanning electron microscopic evaluation results of the enzyme dehaired goat leathers in comparison to the conventionally dehaired goat leathers are similar or even better. These results suggest that the protease extracted from isolated bacterial species has great potential in dehairing of skins thereby reducing the pollution. In essence, the reported research provides a do-only eco-friendly approach for cleaner leather processing utilizing the natural sources derived from wastes.

1. Introduction

Leather processing is an important economic activity in many developing countries, which are dependent on the agro economy. The processes employed in the manufacture of leathers in several developing countries uses conventional method which it is often not optimized for chemicals, energy and water usage[1]. Leather making industry has gained a negative unfriendliness of its tannery wastewater. The pre-tanning process involves 'do-undo' methodologies, such as curing and soaking (dehydration and rehydration), liming and de-liming (swelling and de-swelling), and pickling and de-pickling (acidification and basification)[1]. It contributes to the major amount of pollution approximately 70%[2]. This 'do-undo' logic leads to process inefficiency and ecological imbalances. There is a need to overhaul leather processing by removing these do-undo process logics. This can be achieved by using 'do-only' process logic, where each process involves a specific action without the need for reversing the performed action. This can be achieved by using specific enzyme-based products for a particular action in place of chemicals, which is currently being explored in many areas of leather processing[2].

The most constrained operation from environmental point of view is the dehairing of skin/hide[3]. The conventional method of dehairing involves the use of lime sodium sulfide. Lime produces a poisonous sludge while sodium sulphide is highly toxic and has obnoxious odour. Enzymes are gaining increasing importance in the dehairing process, eliminating the need for sodium sulfide and lime. Enzyme-assisted dehairing process reduces the pollution load to some extent and it is being followed in some parts of the world[3]. It has been speculated that the increase in the production cost due to the application of enzyme can be nullified from the resultant increase in area[3]. A wide range of proteases are used in the leather processing such as neutral proteases in soaking, alkaline proteases in dehairing and acid proteases in bating[2]. Proteases produced by microbial source were more environment-friendly when compared with chemical process and it has tremendous potential in the leather and also other industries[4,5].

Pseudomonas species are being exploited for their ability to produce protease enzyme for dehairing process[6]. In the animal kingdom, fishes are a large group consisting of 24000 species showing wide morphological and habitat variations. Generally, a large amount of fish waste materials around 50% of the weight are generated in fishery processing[7]. This fishery wastes can cause serious environmental problems if disposed improperly. On the contrary, these fishery wastes can be valuable bio-materials, such as proteins, lipids, enzymes, and chitins[8]. Visceral waste has been reported to be a good source of proteins and harbors a microbial population that can produce proteolytic enzymes[9]. Therefore, the present study aims at extracting a crude protease from *Pseudomonas fluorescen*s species from fish visceral wastes and utilize for dehairing of goat skins.

2. Materials and Methods

2.1. Materials

Since this research work involves the study of extent of hair removal, more compact wet-salted goat skins with an average area of 4 square feet per skin were chosen as the raw material. All the chemicals used for processing of goatskin were commercial grade. All chemicals used for the preparation of assay and analysis in the present study were of analytical grade. All microbiological media components were from Hi-media, Mumbai and pure bovine casein used were purchased from Sigma Chemicals. Fish processing industry generates considerable quantities of by-products as wastes that include viscera, shell, scales, fins and frame bones. Fish visceral organs were collected from the local fish market (Chennai) and in stainless steel vessel. Earthen pots were selected for dumping the fish waste with soil. The soil composition included black, red soil and river sand in the proportion of 1:1:2. One kilogram of the mixed soil composition was added to one kilogram of collected fish waste. This was dumped into the earthern pot and kept for seven days. The soil was turned with a thick long wooden stick after every 24 h. Later the soil sample was collected in sterile polythene bag and maintained at 4°C for analyzed.

2.2. Isolation of Bacteria and Enzyme from the Selected Source

Ten grams of soil sample were suspended in 100ml of sterile distilled water and made up to 10^{-8} serial dilutions and vigorously mixed. One ml of each dilution was plated out in casein-yeast extract-peptone (CYP) agar plates, which consist of (g/l): Casein – 10; Peptone – 5; Yeast extract – 1; K_2HPO_4 – 1; $MgSO_4$. $7H_2O$ - 0.2; $CaCl_2$ - 0.1; Na_2CO_3 – 10; and for agar plates 15 g/l was included (Na_2CO_3 was sterilized separately and added to the rest of the medium after cooling) using spread plate technique. The plates were kept for incubation at 37°C for 48 h in inverted positions. Individual colonies were transferred to fresh agar and the plates were incubated at 37°C for 48 h and purified through repeated streaking and it was maintained and stored on CYP slant at 4ºC. The organisms which formed a clearing zone on CYP medium were grown in casein-yeast extract-peptone broth. The cultures were incubated at 30°C with rotary shaking for 48 h. After incubation, about 1ml of culture was transferred to the sterile Petri-plates with CYP agar medium by pour plate techniques. After 48 h incubation at 37°C, protease production was observed as a clearing zone around the colony in

protease positive isolates. Each clear zones of protease positive isolates were inoculated in 250 ml flask containing 50 ml of casein broth. The cultures were grown for 24 h in an orbital incubating shaker at 5000 rpm. The cell-free supernatants were obtained for enzyme assay by reading the optical densities at 660 nm and performing viable cell counts on the total broth. Out of 49 strains, only 24 were found to be protease enzyme producer. The bacterial isolates were plated over nutrient agar medium containing 0.4% gelatin. The zone of growth and hydrolysis of the medium was measured. Out of 24 isolates, 5 strains showed very good zone of clearance. Protease activity were determined for those 5 strains. Depending upon the protease activity, the isolate which produced maximum activity was selected for further experimental studies and maintained nutrient agar slant at 4°C. Identification of selected isolates was studied based on different morphological, physiological and biochemical characteristics. The data was compared as stated by Holt et al.[10] with standard description given in Bergey's Manual of Determinative Bacteriology.

2.3. Growth of Bacterial Culture for Enzyme Production

Among the isolates, *Pseudomonas fluorescens,* which exhibited a prominent clear zone and maximum protease activity, was selected for the study of protease production. The isolate was maintained on CYP agar plate and stored at 4°C. Inocula were prepared by transferring 10 ml pure culture in 50 ml of sterile CYP broth medium and incubated at 30°C on a rotary shaker for 48 h at 5000 rpm. The growth was measured at 660 nm and the culture was harvested by centrifugation at 10,000 rpm for 15 min at 4°C. The cell-free supernatant was used as crude enzyme.

2.4. Enzyme Characteristics

The proteolysis activity was spectrophotometrically assayed by casein digestion method[11]. The protein concentration in the crude extracts of the enzyme was estimated using Lowry's method with bovine serum albumin as standard[12]. The effect of pH was studied by preparing Tris buffer of different pH values as 5, 6, 7, 8, 9, 10 and 10.5 adjusted using 0.1N HCl and 0.1N NaOH. The casein substrate at different pH was prepared by dissolving in appropriate buffer. 1 ml of 1% casein substrate solution at different pH was pipetted out in different test tubes and 0.1 ml of enzyme solution was added. Test tubes were incubated at 30°C and protease assay was carried out as above. The effect of temperature was studied by varying incubation temperature as 20, 30, 35, 40, 50 and 55°C. 1 ml of 1% casein substrate solution was pipetted in different test tubes, maintained at pH 8, and 0.1 ml of enzyme was added. The test tubes were incubated at different temperatures (20, 30, 35, 40, 50 and 55°C) and protease assay was carried out as above.

2.5. Dehairing Studies Using the Crude Enzyme on Goat Skins

2.5.1. Preliminary Trials for Optimization of Enzymatic Dehairing Process

In order to optimize the enzymatic dehairing process, a preliminary study was carried out. Wet-salted goatskins were soaked conventionally. The soaked goatskins were cut into square pieces using sharp knife with the size of 20x20 cm each. The wet weight after soaking was noted for each skin and termed as soaked weight. Paint method of dehairing was followed in all the studies with 5% water. The extracted crude enzyme was varied as 3, 4, 5 and 6% with 0.5% sodium sulfide as well as without (0%) sodium sulfide. No lime was used in all the trials. A solution of each corresponding composition was prepared and then applied on flesh side of the goatskins. All the percentage compositions were based on soaked weight. Then the skins, after application, were piled flesh to flesh and kept for overnight. After 18 h, the skins were dehaired by using a blunt knife. The loosening of hairs and epidermis were observed and visually analysed. Depending upon the extent of hair removal in each case, percentage hair removal was determined based on the basis of the average area without hair out of the total area.

2.5.2. Dehairing: Conventional Vs Enzymatic Process

Wet-salted goatskins were washed and cut into two halves. The right halves of the skin was applied with the optimized enzyme composition, the trial with 100% hair removal efficiency, and treated as experimental skin (ES) while the left halves treated as control skin (CS). Optimized enzymatic dehairing process employs a mixture of 6% extracted crude enzyme, 0.5% sodium sulfide and 5% water based on soaked weight. Control skins were dehaired/limed using a mixture of 3.5% sodium sulfide, 6% lime and 20% water on soaked weight. Paint method of dehairing was followed in both cases by applying the paste on the flesh side, piled and then left for about 18 h. Then, the skins were manually dehaired using blunt knife. The remaining pre-tanning, tanning and post-tanning processes were kept constant for both control (CS) and experimental (ES) skins and the process sequence and chemicals were in line with that meant for garment leathers.

2.6. Evaluation of Pelts and Leathers

2.6.1. Dehaired Goat Pelt Evaluation

Conventional and enzymatic dehaired goat pelts were visually assessed for general appearance, whiteness, epidermis layer, hair roots and hair removal by an experienced leather technologist. Samples were cut, dehydrated and prepared for scanning electron microscopic analysis.

2.6.2. Crust Leather Evaluation

Samples from experimental and control crust leathers were obtained following IUP method[13] for various physical tests. For certain tests such as tensile strength, stitch tear strength and % elongation at break, samples were taken from parallel and perpendicular directions of the backbone of leathers and the average values were calculated. Specimens were conditioned at 20±2 °C and 65±2% R.H. over a period of 48 h. Physical properties such as tensile strength, %elongation at break, and stitch tear strength, were examined following standard procedures[14]. Bulk properties such as softness and bending length were also objectively evaluated following standard procedures[15,16]. Experimental and control crust leathers were assessed for softness, fullness, grain smoothness, uniformity in colour and general appearance by hand and visual examination. The leathers were rated on a scale of 0–10 points for each functional property by four experienced tanners, where higher points indicate better property.

3. Results and Discussion

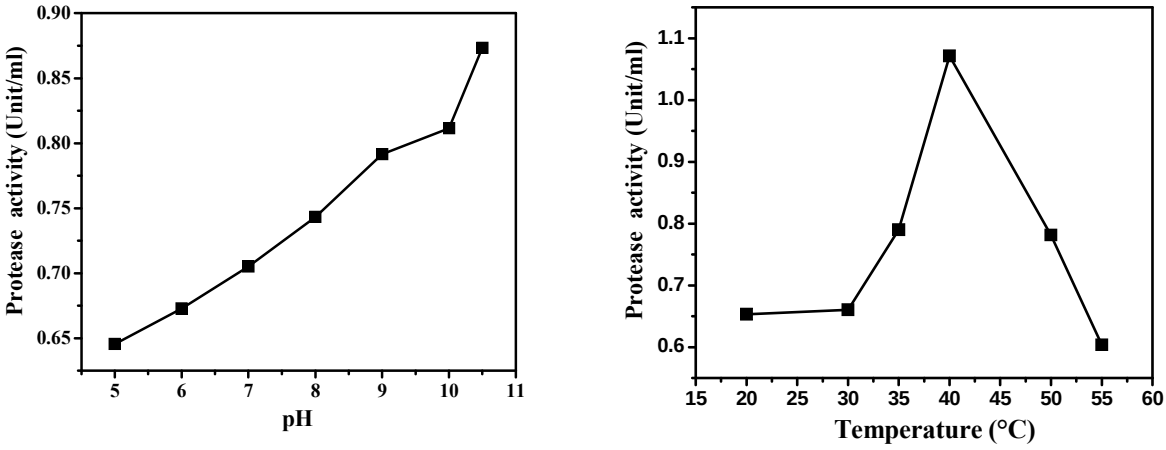
3.1. Isolation, Screening and Identification of Bacteria

Bacterial colonies were isolated from fish waste dumped soil. It was carried out on CYP agar medium by serial dilution technique. A total of 49 isolates were screened for protease production. Out of 49 strains, only 24 were found to be protease enzyme producer. The bacterial isolates were plated over nutrient agar medium containing 0.4% gelatin. The zone of growth and hydrolysis of the medium was measured. Out of 24 isolates, 5 strains showed very good zone of clearance. Protease activity was determined for these 5 strains and results showed that only one strain exhibited maximum protease activity (0.9546 U/ml/min) and hence selected for further study. The Gram staining and motility tests reveal that the selected bacterium is Gram negative and motile with rod shape. The selected bacterium showed positive results for the biochemical characteristics such as catalase test, oxidase test, nitrate reduction test, gelatin hydrolysis test, methyl red test, and citrate utilization test with good growth at 4°C and 41°C. Negative results were obtained for Voges Proskauer test and indole production test. In addition, the selected bacterium showed positive results for fluorescence on King's B medium and Pseudomonas agar for fluorescence. Hence, selected bacterium has been identified as *Pseudomonas fluorescens*, as shown in Fig. 1. The activity of the enzyme prepared from the subculture of *Pseudomonas fluorescens* is found to be 0.9546 U/ml/min and the specific activity is 1.2197 U/mg/ml.

Fig. 1. Subcultured
Pseudomonas fluorescens

3.2. Effect of pH and Temperature on the Activity of Protease

Enzyme activity at various pH values such as 5, 6, 7, 8, 9, 10 and 10.5 was analyzed and the results are shown in Fig. 2a. It is seen that the protease activity increases as pH increases and attains maximum at pH 10.5. The effect of temperature on the activity of protease is shown in Fig. 2b. It is seen that the optimal temperature for maximum protease activity is 40°C for the enzyme derived from *Pseudomonas fluorescens*. There was a significant reduction in protease activity for the enzyme derived from *Pseudomonas fluorescens* above or below 40°C.



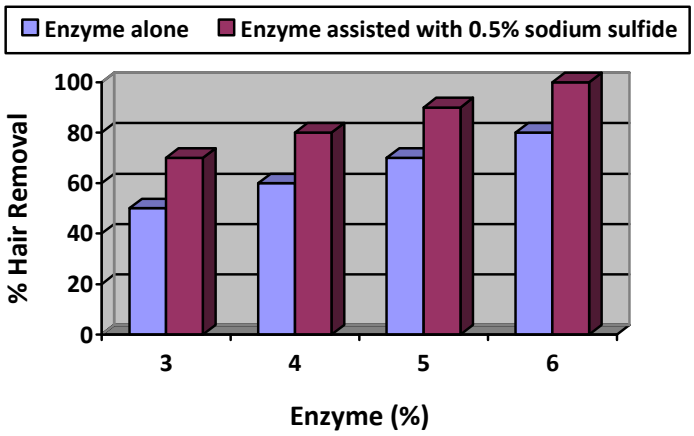
(a) (b)

Fig. 2. Effect of (a) pH and (b) temperature on the protease activity derived from *Pseudomonas fluorescens*

3.3. Dehairing Efficiency of Protease Derived from *Pseudomonas fluorescens* on Goat Skins

The effect of enzyme concentration for enzyme-only and enzyme-assisted with less sulfide deharing on goat skin was studied and the percentage of hair removal is shown in Fig. 3a. It is seen that increase in the concentration of crude enzyme results in a significant increase in the percentage hair removal of goat skins. Sulfide-free dehairing (3 to 6% enzyme) results in 50 to 80% dehairing only, on the other hand enzyme-assisted with less sulfide dehairing (0.5% sodium sulfide with 3 to 6% enzyme) results in 70 to 100% dehairing. Specifically, 6% crude enzyme along with 0.5% sodium sulfide results in complete dehairing without employing any lime, which was considered for further studies. This could be due to the fact that the presence of sodium sulfide not only improves the dehairing efficiency but also enhances the activity of crude enzyme by increasing the pH towards

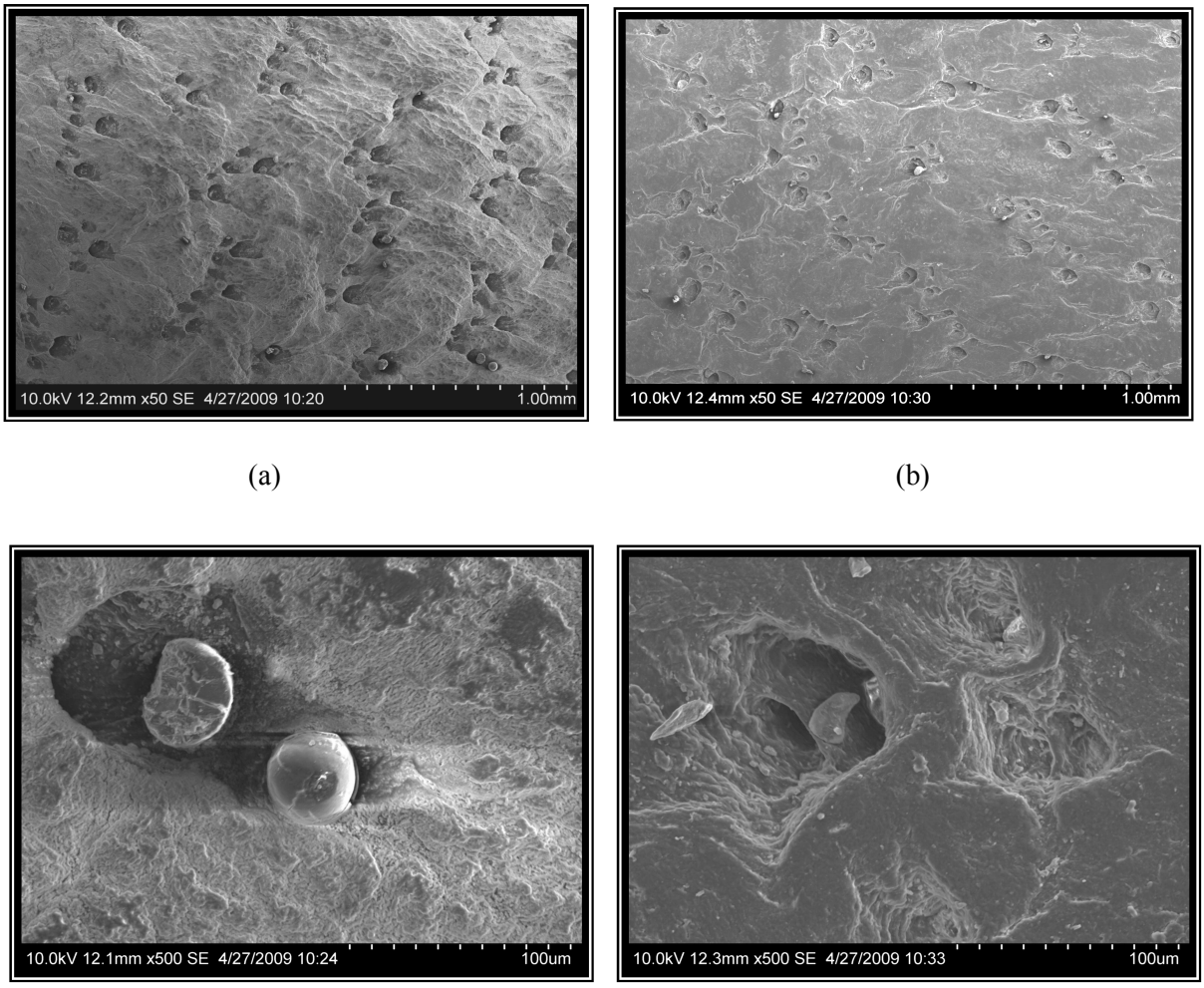
10.5. Based on the results obtained from preliminary investigations, trial resulted in 100% dehairing was considered as optimized enzyme composition and chosen for further study. The optimized enzymatic dehairing recipe was compared with the conventional dehairing process. As seen from Fig. 3b, visual inspection showed that the pelt produced by optimized enzyme composition exhibited comparable hair removal, better general appearance and better whiteness when compared to conventionally dehaired goat pelt. The scanning electron micrographs of pelt samples from conventional and enzymatic dehairing processes are shown in Fig. 4.



(a)

(b)

Fig. 3. (a) Extent of dehairing for the selected enzyme compositions derived from *Pseudomonas fluorescens* and (b) pelt after manual dehairing using blunt knife



(a) (b)

(c) (d)

Fig. 4. Scanning electron micrographs showing the grain surface of pelt after conventional (a and c) and enzyme based (b and d) dehairing at low and higher magnifications, respectively

Conventional pelt sample shows a white surface indicating the deposition of lime (Fig. 4a). It also shows certain bigger particles in the hair pores, which may be the unremoved hair root or lime particles. On the other hand, enzyme treated sample shows a clear surface without any deposition of foreign particles or grain damage, indicating complete hair removal (Fig. 4b). As shown in Fig. 4c, higher magnification photograph of conventional sample exhibits the presence of hair root or lime particles on the hair pore, confirming the observations made at lower magnification. While the enzyme treated sample shows clean hair pores without any major deposition of foreign particles, indicating complete hair removal (Fig. 4d). These results indicate that the crude enzyme developed from fish visceral wastes can remove the hair completely when applied with less amount of sodium sulfide and the dehairing efficiency is comparable to that of conventional dehairing.

3.4. Physical and Bulk Properties of Conventional and Experimental Crust Leathers

Control and experimental crust leathers were assessed for various bulk properties by hand and visual examination. The average of the rating for the leathers corresponding to control and experiment is given in Fig. 5 for each functional property. Higher numbers indicate better properties. It is seen that

the leathers derived from enzymatic dehairing processes exhibit similar or better bulk properties such as softness, grain smoothness, uniformity in color and general appearance compared to the conventionally dehaired leathers. Fullness of the experimental leathers is slightly lower when compared to the conventionally dehaired leather. The overall performance of both conventional and enzyme treated leather was comparable. It is seen that the enzyme treated leather possesses a little higher softness value to that of conventional leather. This result is comparable to the results obtained by using a commercial protease[3], indicating the ability of enzymes in the softening of leather.

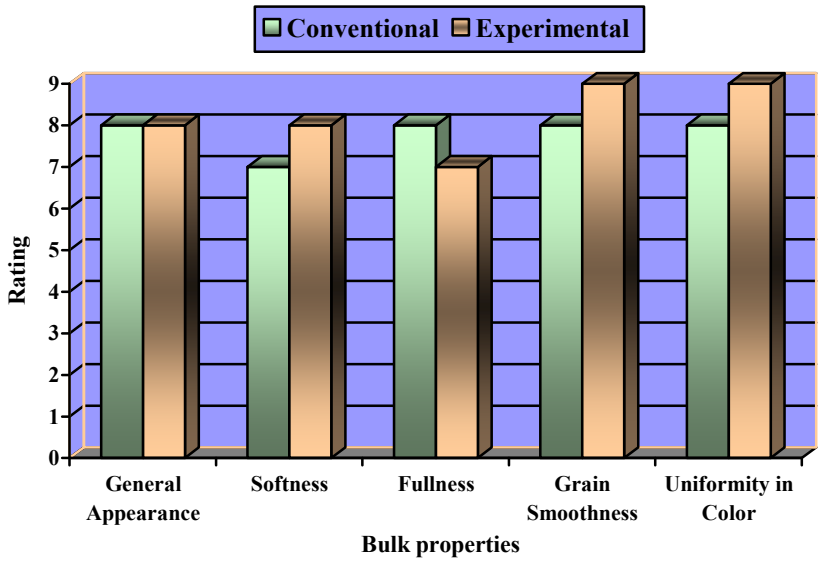


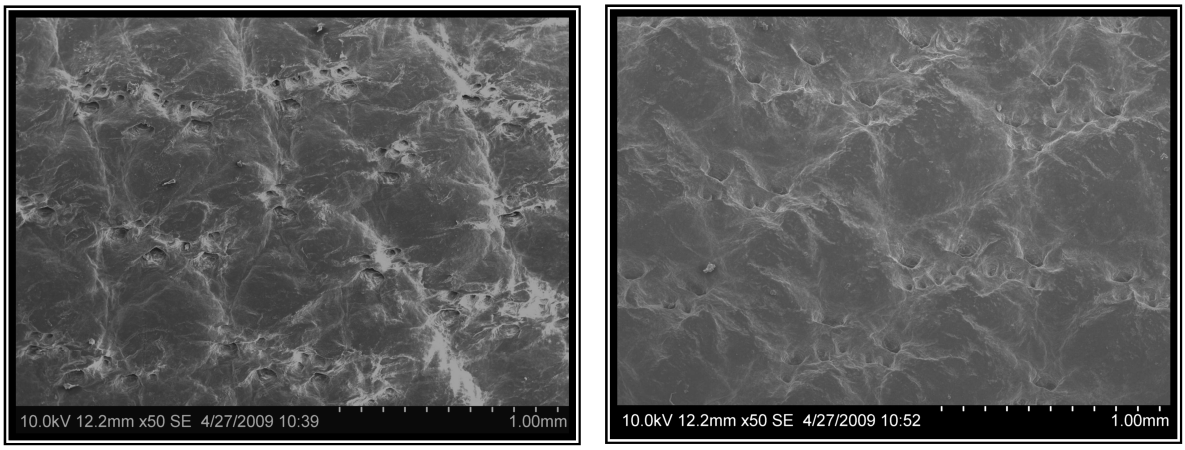
Fig. 5. Bulk properties of the conventional and experimental leathers

The strength properties of the control and experimental crust leathers were measured and the results are shown in Table 1. It is observed that the conventional and enzymatically dehaired goat leathers meant for garment production have statistically comparable tensile strength, percentage elongation at break and double-hole stitch tear strength values. In other words, the strength properties of the leathers did not adversely affect due to the enzyme application in comparison to the lime-sulfide application in control process. It is interesting to note that the conventional and enzymatically dehaired garment leathers possess statistically different softness values. Higher softness value indicates better softness. Results suggest that the leathers processed by enzyme application have slightly higher softness value of 6.1 mm and hence they are much softer when compared to that of conventional leather samples, which has a softness value of 5.9 mm. These results are in agreement with the bulk property evaluation. It is observed that the conventional and enzymatic dehaired goat leathers used for making garment possess statistically different bending length values. Lower bending length value indicates better bending.[17] It is seen that the enzymatically dehaired leather has slightly low bending length value and hence better bending and draping[17] when compared to that of conventional garment leather which has fairly high bending length value. These results indicate that the enzymatic garment leather has better comfort properties required for clothing application.

Table 1. Strength, softness and bending properties of the conventional and experimental leathers

Treatment Process	Tensile strength (N/mm^2)	% Elongation at break	Double-hole stitch tear strength (N/mm)	Softness (mm)	Bending length (mm)
Conventional dehairing	22.5±3.5	59±2	135.5±4.1	5.9±0.1	33.8±0.6
Enzymatic dehairing	21.4±1.9	58±3	127.5±5.2	6.1±0.1	31.7±0.4

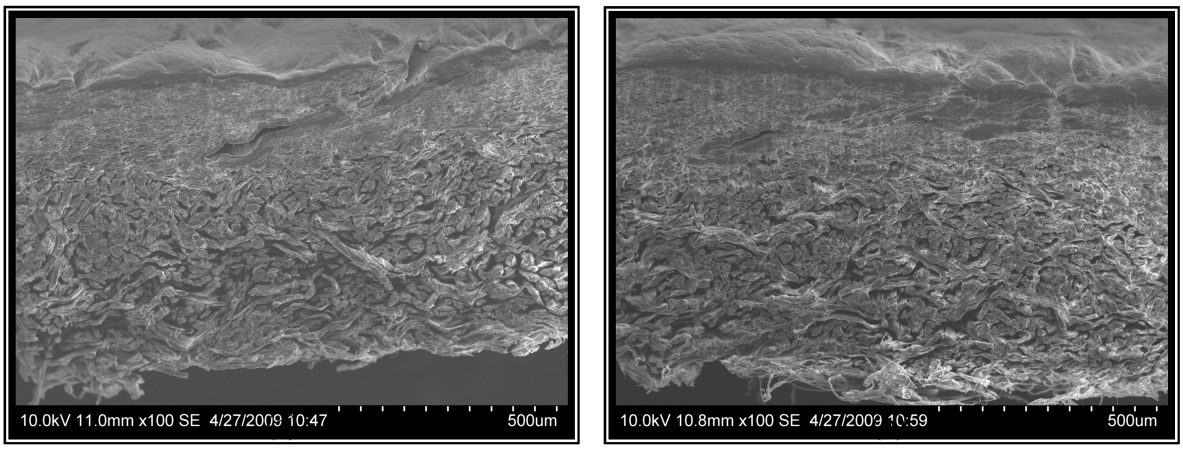
The scanning electron micrographs of conventional and enzymatically dehaired goat crust leathers showing the grain surface are presented in Fig. 6. Both conventional and enzyme treated goat leather samples show clear grain surface without any deposition of foreign particles, indicating complete hair removal. The lime deposition observed after dehairing in the conventional sample (Fig. 4a) was removed during subsequent deliming and pickling processes. It is also evident that the enzyme treatment did not lead to any grain damage due to the action of enzyme on grain structure (Fig. 6b). This may be due to the careful choice of bacteria, which yielded a protease devoid of collagen specific enzymes (such as collagenase), as well as other process control measures.



(a) (b)

Fig. 6. Scanning electron micrographs showing the grain surface of crust leathers processed through conventional (a) and enzyme based (b) dehairing

The scanning electron micrographs showing the cross section of conventional and enzymatically dehaired goat crust leathers are depicted in Fig. 7. Both conventional and enzyme treated goat leather samples exhibit a comparable fibre bundle structure with moderate fibre opening across the cross section. This implies that the extent of fibre opening during the reliming process for enzymatically dehaired pelt is sufficient to produce quality garment leathers in comparison to the conventional leathers, which were treated by a double liming process (liming-reliming). These results are in agreement with the objective and subjective softness assessment, which is generally regarded as an indirect measure for fibre opening.



(a) (b)

Fig. 7. Scanning electron micrographs showing the cross section of crust leathers processed through conventional (a) and enzyme based (b) dehairing

4. Conclusions

An attempt has been made to exploit the fish visceral waste as a bacterial source for possible production of protease for application in leather processing. The fish visceral waste were dumped in a select soil composition and decomposed. This soil sample was utilized to culture bacterial colonies from which a proteolytic bacteria was screened and isolated. The selected proteolytic bacterium was identified as *Pseudomonas fluorescens* using biochemical characterizations and the activity of the extracted crude enzyme was analyzed. The activity of the crude enzyme was maximum at pH 10.5 and temperature at 40˚C. The efficiency of the crude protease as a dehairing agent was analyzed on a goat skin and compared with the conventional dehairing procedure. The optimized procedure for enzymatic dehairing of goat skin was found to be 6% crude enzyme, 0.5% sodium sulphide and 5% water at room temperature by painting on flesh side for 18 h. SEM analysis of goat pelt proved that enzymatic dehairing with the crude protease was efficient in the hair removal from goat skin. Most of the physical and bulk properties of the garment leathers from enzymatic dehairing are similar or comparable to the conventionally dehaired goat garment leathers. Especially, enzymatically dehaired garment leathers have better comfort properties such as softness and bending length (drape properties) required for clothing application in comparison to control leathers. The results of this study suggest that the extraction of crude protease from *Pseudomonas fluorescen*s may play a significant role in dehairing of goat skins, if appropriately studied on a pilot scale.

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