Call for Papers for the 104th Annual Meeting of the American Leather Chemists Association June 19-22, 2008

If you have recently completed or will shortly be completing research studies relevant to hide preservation, hide and leather defects, leather manufacturing technology, new product development, tannery equipment development, leather properties and specifications, tannery environmental management, or other related subjects, you are encouraged to present the results of this research at the next annual meeting of the Association to be held at the Grandover Resort and Convention Center, Greensboro, NC June 19-22, 2008.

Abstracts must be submitted to the Chair of the Technical program:

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Each abstract should begin with the title in capital letters, followed by the authors' names. The name of the speaker should be denoted by an asterisk, and contact information should be provided that includes an e-mail address. The abstract should be no longer than 300 English words and preferably furnished as a Microsoft Word or Adobe PDF document.

Manuscripts based on the presentation, in publication-ready form, are expected by May 15, 2008. They should be sent in electronic format (either as an email attachment or on a CD-R) by that date to:

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Presentation at the convention will be limited to 25 minutes. In accordance with the Association Bylaws, papers covering such presentations are to be submitted to the *Journal of the American Leather Chemists Association* for publication consideration. Those papers are not to be published elsewhere, other than in abstract form, without permission of the *Journal* Editor, Robert F. White.

Microbial Degradation Of Horn Meal With *Bacillus Subtilis* And Its Application In Leather Processing: A Two Fold Approach

by
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Abstract

Horn meal hydrolysate, obtained by high steam pressure treatment of raw horns and hoofs of cattle and buffalo, yields a mixture of water soluble peptides by microbial degradation using native strain of *Bacillus subtilis*. It is designated as Bacterial Degraded Horn Meal Hydrolysate (BDHH). BDHH stores well and does not putrefy when left at ambient conditions of temperature (32±3°C) and relative humidity (40-80% RH). This material is employed with great success in leather tanning process to improve the exhaustion of chrome and to fill the void within the tanned collagen fibers during retanning operation. Use of BDHH in leather processing could cut down consumption of chromium salts in chrome tanning by considerably reducing the discharge of chromium in the waste liquor from 30-35% to less than 10%, thus saving cost on chromium salts and reducing the pollution load in commercial tanning operations. Filling of tanned leather with BDHH in retanning operation upgrades the crust leather.

Resumen

El hidrolizado de harina de cuerno, obtenido mediante el tratamiento con vapor a alta presión de cuernos crudos y pezuñas de ganado vacuno y de búfalo, resulta mediante la degradación microbiana usando la acción natural del bacilo subtilis, en una mezcla de péptidos solubles en agua. Se lo designa como Hidrolizado de la Harina de Cuerno por degradación bacteriana (BDHH). El BDHH se almacena bien y no se pudre cuando se deja a condiciones de temperatura ambiente (32±3°C) y de humedad relativa (HR 40-80%). Este material es empleado con gran éxito en el proceso del curtido de cueros para mejorar el agotamiento del cromo y para llenar el vacío dentro de las fibras de colágeno curtidas durante la operación de recurtido. El uso de BDHH en el proceso del cuero podría reducir el consumo de sales del cromo en el curtido al cromo considerablemente reduciendo la descarga de cromo en el efluente de un 30-35%, a menos del 10%, ahorrando de esta manera en los costos de las sales de cromo y la reducción de la carga contaminante en operaciones de curtidos comerciales. El relleno del cuero curtido con BDHH en la operación de recurtido mejora el cuero semi-terminado.

Introduction

In leather processing tanning is an important step, which converts putrescible skin collagen into stable leather. At present 90% of the tanning processes[1] use chromium tanning salts due to the versatility of the tanning system to produce different types of leathers with required properties.[2,3] Tanning system is coming under increased pressure from all over the world due to environmental pollution and use of toxic materials.[4-8] In chrome tanning the exhaustion of chromium in the tanning bath does not exceed 60-65% in commercial tanneries.[9,10] Unused chromium present in the discharge contributes 29,000-57,500[11] mg/l total dissolved solids (TDS) in waste water causing environmental pollution. Today in most of the countries the discharge limit of chromium in the waste stream should not exceed 2.0 ppm.[12] This necessitates treatment of discharge in Common Effluent Treatment Plant (CETP) before it let out for usage. This leads to increase in cost of leather processed in tanneries.

We have another problem of biological solid waste let out by the byproducts industry. Keratin wastes like hair, feathers, nails, horns, hoofs, scales and wool are increasingly accumulating in the environment generated from poultry and meat processing plants, slaughterhouses, tanneries and other industries. Presence of disulfide bonds in keratins hinders their degradation by commonly known proteolytic enzymes like trypsin, pepsin and papain.[13,14] By chemical processing[15,16,17,18] we can convert those keratinous waste into useful soluble materials but again chemical processing causes environmental pollution. To overcome this situation we are looking into alternative methods to advantageously treat this material. Microbial treatment can efficiently degrade this material by numerous bacteria, actinomycetes and fungi by synthesis of keratinolytic

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proteases-keratinases.[19] Keratinases from *Bacillus sp.* particularly *B. licheniformis* and *B. subtilis* have been extensively studied for the effective degradation of feather.[20,21] Biodegradation by these organisms represents an improved method for utilization of these waste materials into useful products.

Keratinases are robust enzymes with a wide temperature and pH activity range and are largely serine or metalloproteases. Among all proteases, they standout separately since they attack the keratin residues and convert them into degradative and cost effective products from keratinous waste like feather, steam hydrolyzed horn powder and others. These products have wide application in many industries including leather tanning industry. With this soluble material obtained by microbial treatment we are trying to look for alternative solutions, particularly increasing chromium uptake in tanning using microbial route to solve this problem. In the present investigation, we are reporting the degradation of keratin hydrolysate by the native bacterium and utilizing it in chrome tanning to improve the uptake of chromium and in retanning to get the desired level of fullness, feel and filling properties.

MATERIALS AND METHODS

Isolation and Screening of Proteolytic Organism

The bacterial strains were isolated from the horn meal and evaluated for their proteolytic activity in skim milk agar. In brief, 1 g of scrapped horn meal taken in 100ml of physiological saline solution (10^{-2}) and mixed vigorously for few minutes in a cyclomixer. Serial dilution was made up to (10^{-7}) and 1ml of aliquots from each dilution was transferred to appropriately marked skim milk agar plates and spread over the agar surface. The plates were incubated at 37°C for 24- 48 hrs. A qualitative screening for the proteolytic activity of the isolates was indicated by growth and clear zone around the growth on skim milk agar. The screened bacterial isolates were grown in nutrient broth (Hi-Media Pvt. Ltd., Mumbai, India) for overnight at 37°C. They were adjusted to yield approximately 1.0 x 10^5 CFU/ml. 5ml of broth culture was inoculated into the mineral salt medium (NH_4Cl - 0.5g, NaCl - 0.5g, K_2HPO_4 - 0.3g, KH_2PO_4 - 0.4g, $MgCl_2.6H_2O$ - 0.24g, Yeast extract - 0.1g, Distilled water – 1000ml, pH 7) with horn meal (1% w/v) and incubated at 37°C with rotary shaking for five days. After incubation, the solubilization of the horn meal was measured by estimating the amount of insoluble horn meal through filtration. The isolate, which possess higher keratinolytic activity was sub cultured and stored as glycerol stock at -20°C. The identification of isolate was done according to colony morphology, staining procedures and other biochemical tests.

Preparation of Horn Meal

Raw horns of slaughtered cattle and buffaloes collected from the local slaughterhouse at Perambur Chennai were subjected to high steam pressure (40psi) in a wet rendering plant (FMC, Australia) for three hours (raw horn water ratio 100:30 w/v). The resulting material was dried in a dryer (BHL, Ahmedabad) and pulverized in pulverizer (FMC, Australia) to get horn meal. 100 Kg raw horns give 60 Kg of horn meal (average yield). Horn meal was collected from the pilot plant of Central Leather Research Institute and washed twice with distilled water to remove the rough dirt and soils from the material and dried at 60°C overnight. It was kept at room temperature and used as raw material.

Inoculum Preparation

The bacterial culture was grown in nutrient broth (Hi-Media Pvt.Ltd., Mumbai, India) for overnight at 37°C and adjusted to yield approximately 10^5 CFU/ml was used as an inoculum.

Biodegradation of Horn Meal Hydrolysate

1000ml of Mineral salt medium (NH_4Cl - 0.5g, NaCl - 0.5g, K_2HPO_4 - 0.3g, KH_2PO_4 - 0.4g, $MgCl_2.6H_2O$ - 0.24g, Yeast extract - 0.1g, Distilled water – 1000 ml, pH 8) with 10g horn meal was prepared and sterilized by autoclave at 121°C for 15 minutes. 50ml of seed inoculum was added to the medium and incubated for 5 days at 37°C with constant shaking at 120 rpm). After incubation, the medium was centrifuged at 10000 rpm for 10 minutes at 4°C and the supernatant containing BDHH, was concentrated (30% solid content) in a water bath and used in leather processing.

(Note): The process was up scaled to 300g material (horn meal) keeping all the condition identical as described above.

Beamhouse procedure for goat skin

Goat skins were used to test the efficacy of horn meal hydrolysate on chromium exhaustion. Three sets of trials were carried out. In each set six wet salted goat skins were taken from CLRI cold room and marked as 1L 1R, 2L 2R, 3L 3R, 4R 4L, 5,6. 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.

Conventional chrome tanning procedure

Pickled skins 1L, 2R, 3L, 4R and 5 were processed using 8% Basic Chromium Sulphate (BCS) having 23% Cr_2O_3 content 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.

BDHH chrome tanning procedure

Pickled skins 1R, 2L, 3R, 4L and 6 were processed using 8% BCS and 50% pickle water for 90 min. Then 50% water

TABLE 1
Percentage Exhaustion of Cr_2O_3

Trial No.	% Exhaustion of Chromium as Cr_2O_3		% of Cr_2O_3 in leather (dry weight)	
	Control	Experiment	Control	Experiment
1	67±2	91±2.5	3.2±0.3	4.2±0.5
2	65±3	92±2	3.0±0.2	4.4±0.3
3	68±2	93±2	3.2±0.3	4.4±0.2

was added and run for 30 min. 3% BDHH was added and the drum was run for 45 min. Then 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.

Determination of Chromium

The chromium content in the spent liquor obtained in each of the tanning experiments as well as in the leather was determined by acid digestion method.[22]

Use of BDHH in Post Tanning (Retanning)

The hydrolysate that has rich in protein content is used as filler in retanning of chrome tanned leathers. Shaved wet blue goat leathers having 1mm thickness were used as raw material for post tanning trials. The leathers were washed and neutralized to pH 5.2 and washed twice. Control leathers were retanned using 5% commercial protein filler (% based on shaved weight) and experimental leathers were with 5% BDHH for 30 min. The remaining procedure (dyeing and fatliquoring) was common for both control and experimental leathers. 2% acid dye and 9% commercial fatliquoring agent was used and finally fixed with formic acid.

Scanning Electron Microscopy Analysis

In order to study the effect of BDHH on the structural characteristics of the leathers produced, scanning electron microphotographs of control and BDHH treated leathers (both tanning and retanning trials) were compared. The samples measuring 5mm x 2mm were cut from the official butt portion.[22] The samples were mounted vertically on aluminum stubs using an adhesive. These were then coated with gold using an Edwards E-306 sputter coater. Thickness of coating was adjusted to minimum level required to prevent charging. 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 an accelerating voltage.

Physical Analysis and Visual Assessment

The samples for physical testing were cut from both BDHH and commercial protein filler treated goat crust leathers (retanning trials) according to the official sampling position.[23] The samples were conditioned to the required relative humidity of 65±2% at 20±2°C for 48 h as per IUP 3.[24] The tensile and tear strengths were measured as per the standard procedures.[25,26] 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 softness, feel and grain smoothness.

RESULTS AND DISCUSSION

Isolation and Screening of Proteolytic Organism

Among the isolates studied, six were found to produce good growth with clear zones on skim milk agar. Based on the morphological and biochemical parameters the isolate *Bacillus subtilis* was identified and considered to be an effective organism for the degradation of horn meal reported first time in our study. Regarding industrial applications, microbial conversion of horn meal wastes is a potential technique for degradation and utilization of horn meal as a hydrolysate in the cost-effective, environmentally benign leather processing. All keratinolytic enzymes act as proteases and are active on keratin.[27] However, the exact mechanism of keratinolysis is not fully understood. Numerous reports are available on the use of keratinases and their products to degrade feather keratin.[28-31] In our investigation, the native isolate released high levels of soluble protein into the medium using horn meal as the sole source of carbon and nitrogen is successfully employed in leather tanning processes.

Use of BDHH on Chromium Exhaustion

Keratin hydrolysate generate additional carboxylic groups which have high affinity for Cr(III). The exhaustion of chromium in the tanning process is above 90% by the use of bacterial degraded horn meal hydrolysate. The results are presented in Table 1. It is seen that the exhaustion of chromium in each experimental trial is above 90%. Also the percentage fixation of Cr_2O_3 in the experimental leather is more than control. The peptide obtained from the hydrolysis of horn meal with *Bacillus subtilis* has able to cross link with both Cr(III) and collagen. Fixation of keratin complex in leather enhances uptake of Cr(III) in leather resulting into enhanced uptake of Cr(III). Besides, fixation of soluble keratin hydrolysate in leather generate additional carboxylic groups which have high affinity for Cr(III).

Effect of BDHH on Collagen Fibers

The scanning electron microphotographs of leathers obtained by the use of BDHH in chrome tanning and in post tanning

TABLE 2
Physical Testing Data (derived from retanning trials)

Properties	Control	Experiment
Tensile Strength kg/cm^2	208±3	210±4
% Elongation	70±3	69±2
Tear Strength kg/cm	40±3	42±2

TABLE 3
Visual Assessment Data (scale 1-10) (derived from retanning trials)

Properties	Control	Experiment
Fullness	8±1	9±0.5
Grain smoothness	8±1	8.5±0.5
Softness	8±0.5	8±0.5

operations showing their cross section at a magnification of 500x are given in Figures 1 and 2 respectively. From the figures it is evident that the fiber structure of BDHH tanned and retanned crust leathers do not show any adverse physical change and it is comparable to that of control. In Figure 2 BDHH retanned collagen fiber bundles seems to be compact in nature compared with commercial protein filler retanned leather. Fiber compactness is an indirect measure of fullness which is clearly evident from the visual assessment data of experimental leather.

Experiment (BDHH treated) **Control**

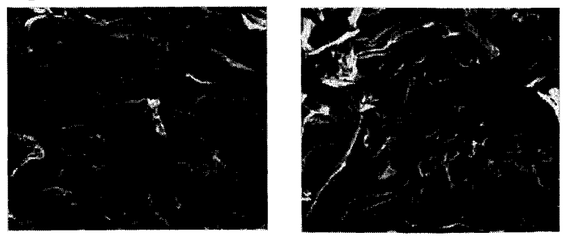

Figure 1: Effect of Horn Meal Hydrolysate on Collagen Fibres (tanning trial)

Experiment **Control**

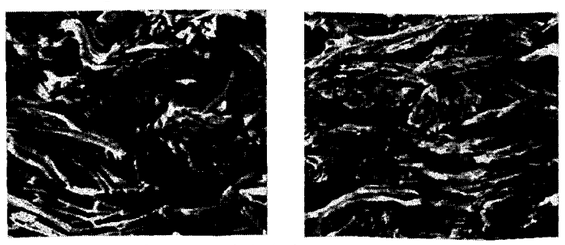

Figure 2: Effect of Horn Meal Hydrolysate on Collagen Fibres (retanning trial)

Physical Testing and Visual Assessment Data

The physical characteristics and visual assessment data for both control and experimental leathers are given in Tables 2 and 3 respectively. The values are comparable showing no adverse effects due to the treatment of horn meal hydrolysate. The use of bacterial degraded horn meal hydrolysate in retanning process influences lubricating effect that enhances the grain smoothness and softness characteristics of the leathers, which is evident from the visual assessment data.

Economic Feasibility in the Preparation of Horn Meal

Cost of horn meal production (Large scale- above 100-150 tons/month) in India roughly comes to Rs.7-8 per kg (manufacturer & exporter: P. Subbraj & Company No.5, 1st Street, Sylvan Lodge Colony, Kilpauk, Chennai-600007, India). The selling price of this material is around Rs.13 per kg. This material is exported from India to many countries in Europe and also to Australia. Based on this calculation we feel horn meal hydrolysate would be a commercially viable product if manufactured in any part of the world.

CONCLUSION

We have proposed a simple method for effective utilization of horn meal in leather processing. BDHH will substantially improves the fixation of chromium and thereby reduce pollution load and processing costs. The microbial degradation of horn meal hydrolysate is a better option compared to chemical methods. The use of keratin hydrolysate in leather processing has two-fold advantage. Initially the bio waste is converted into keratin hydrolysate, hydrolysate is used as exhaustive aid in chrome tanning to reduce the pollution load and finally it is used as filler-cum-retanning agent in retanning process to replace existing retanning-cum-filling material used by the leather industry. In conclusion, this study may provide complementary steps towards the establishment of environmentally friendly technology for the treatment of keratin wastes as well as in leather tanning process.

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