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Isolation and identification of proteolytic *Bacillus pumilus* from tannery lime effluent and its dehairing activity

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Abstract
Enormous amount of inorganic sulfides and lime employed for dehairing of raw hides in the tanneries contribute to the major proportion of chemical load of the tanners, resulting in the discharge of sulfide rich liquors out of the tanneries. An attempt has been made to isolate a proteolytic bacteria from tannery lime effluent. Isolate (T5) that showed the largest zone of lysis on skim milk agar plate was identified as *Bacillus pumilus* through MALDI-TOF. Vigorous hair removal from hides was observed when they were soaked in the culture supernatant of *Bacillus pumilus* for 24 hrs, thus proving its ability of unhairing the hides by its protease enzyme. The enzyme was partially purified by acetone precipitation for further studies.

Keywords: *Bacillus pumilus,* De-hairing, Protease enzyme, Sulfide, Tanneries.

1. Introduction
Leather industry is a pollution-intensive industry, due to the usage of large amount of surfactants, hazardous chemicals, and solvents for processing of raw hides. Conversion of raw hides into finished leather products involves many sequential processes, initiating from collection of raw hides and its storage, beamhouse (limeyard) operations, followed by tanyard and post-tanning operations (also called as wet finishing) and finally dry finishing operations which involves mechanical finishing. Unhairing of hides is one of the most important processes in leather making, in which, chemicals such as lime and alkali sulfides are used, to remove interfibrillary proteins, hair roots and hairs from the hides, in large rotatory drums. This process emits large amount of sulfides (in the form of hydrogen sulfide), resulting in sulfide-containing liquors in the wastewater effluent. This also leads to noise pollution as well as emission of sulfide odours, destroying the health of the tanners and the nearby public.
As an efficient but expensive alternative, microbial proteases are gaining importance in the tanneries. Enzymatic dehairing is suggested as an environmentally friendly alternative to the conventional chemical process [6]. Proteolytic enzymes are more efficient in enzymatic dehairing than amylolytic enzymes [5]. Bacterial protease does this work perfectly, but the major drawback with them is their high production cost when compared with conventionally employed dehairing chemicals' cost. In this present paper, an attempt has been made to isolate, and characterize protease producing microorganism from tannery lime effluent and to check its enzymatic de-hairing activity on raw leather hides. With this aim in mind, the main objectives of this study are: i) to isolate proteolytic bacteria from tannery lime effluent and its characterization using MALDI-TOF; ii)
Production of crude protease from proteolytic bacteria through fermentation; iii) to examine the direct de-hairing activity of the proteolytic bacteria and IV) partial purification of protease enzyme.

2. Materials and Methods
2.1 Sample Collection
For the isolation and identification of proteolytic bacteria, tannery lime effluent sample was collected in sterile sampling bottles from EKM Leather Processing Company, Khaleel Tanning Company, in Erode District of Tamil Nadu, India. The samples were transferred immediately to the laboratory for further analysis.

2.2 Isolation of Microorganisms from Lime Effluent
Serial Dilution was performed to enumerate the total number of viable cells present in the lime effluent. About 1ml of the sample was added to 9ml of sterile distilled water and this suspension was serially diluted. About 0.1 ml from each dilution was spread plated onto sterile nutrient agar plates and incubated at 37 °C for 24 hours. Total number of viable cells is counted using the following formula –

$$\text{Total no. of viable cells} = \text{Average no. of colonies} \times \frac{\text{Dilution factor}}{\text{Inoculum size}} \left\{ \frac{\text{cfu}}{\text{ml}} \right\}$$

2.3 Screening of Proteolytic Isolates
Skim milk agar was prepared and the above colonies were streaked on milk agar plates for testing the caseinolytic activity of the organism. Isolates were inoculated onto plates and incubated at 37 °C for 24 h. Strains producing clearing zones in this medium were selected [2].

2.4 MALDI-TOF Analysis of the Proteolytic Bacteria
Strain that produced the largest clear zone on skim milk agar was further identified using MALDI-TOF by an instrument named MALDI Biotyper. An isolated colony was mixed with matrix and added to MALDI-TOF project list. Software generated a spectrum, which was then instantly matched

against reference library to give identification, with the help of the score value obtained.

2.5 Crude Protease Production and Its Direct De-hairing Activity

Submerged fermentation was performed by inoculating pure culture of isolate into the production medium [4]. The organism was grown in nutrient broth at 37 ºC for around 3 days. Then it was centrifuged at 5000 rpm for 15 minutes. The cell free supernatant acted as crude enzyme. The detergent washed cow skin was immersed in crude enzyme of the isolate to observe its enzymatic de-hairing capability. Sodium chloride (1%) was added to prevent the growth of spoilage microorganisms. Nutrient broth was used as control.

2.6 Partial Purification of Protease

The cell free extract from fermentation broth was partially purified by acetone precipitation method [9]. Protease was precipitated by pre chilled acetone (30-80%) fractionation. The acetone was added to the cell free extract in 3:1 ratio and incubated for 60 min at -20 °C. The contents were subjected to centrifugation at 10000 rpm for 10 min. The supernatant was discarded carefully and the pellet was dissolved in Tris-acetate buffer (pH 7).

3. Results

In the present study, an efficient proteolytic bacteria was isolated from tannery lime effluent and de-hairing of tannery hides using the crude protease enzyme of the isolate was examined.

3.1 Isolation of Microorganisms from Lime Effluent

The microbial load of the lime effluent from the tannery industry was determined by performing serial dilution and colonies observed in each dilution were counted and expressed in terms of cfu/mL. Out of numerous colonies observed, few distinct non-overlapping colonies were selected and sub cultured for further screening.

3.2 Screening of Pro teolytic Isolates

Three strains out of fifteen were able to form lysis zones on skim milk agar plates. Isolates T1 and T3 showed a mild zone of lysis whereas Isolate T5 showed the largest zone of lysis within 24 hours of incubation on skim milk plates, thus proving its efficiency of vigorous protease production. Isolate T5 was subjected to identification using MALDI-TOF.

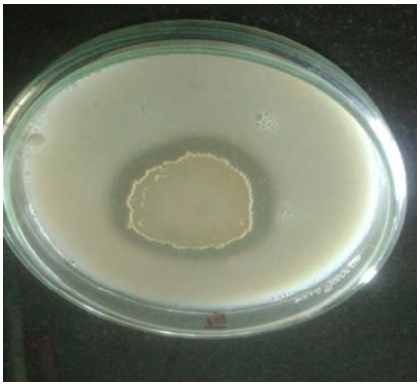


Fig 3.1: Zone of hydrolysis of *Bacillus pumilus* on skim milk agar plate.

3.3 MALDI-TOF Analysis of the Proteolytic Bacteria

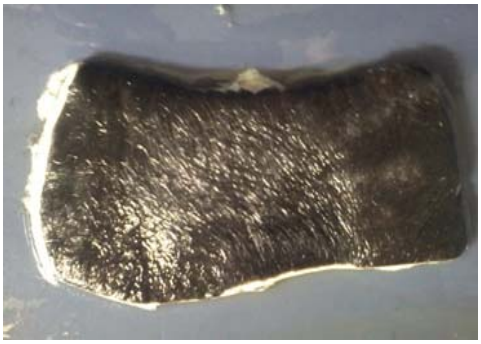
Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry was performed to identify the selected bacteria. Based upon the score value, the organism (T5) was identified as *Bacillus pumilus*.

Table 3.1: MALDI-TOF identification result of the isolate (T5)

Analyte ID	Organism (Best match)	Score value
T5	*Bacillus pumilus*	1.556

3.4 Direct De-hairing Activity of the Enzyme

The de-hairing activity of the T5 culture filtrate was tested on small uniform pieces of tannery raw hides. Hides were soaked in the culture supernatant that acted as a crude enzyme, which influenced directly on the hairs of the hides during incubation. After 12 hours of incubation, the hides were observed to be softer than their native form and upon gentle rubbing, mild removal of hairs was observed. Vigorous removal of hairs from hide was found within 24 hours of incubation, thus proving the efficiency of *Bacillus pumilus* protease enzyme to act on the keratin protein present on the hairs of the hides.



Control

After incubation in T5 supernatant for 24 hours

Fig 3.2: Direct De-hairing Activity of the enzyme isolated from *Bacillus pumilus* (T5)

Table 3.2: Direct De-hairing Activity of the enzyme isolated from *Bacillus pumilus* (T5)

Incubation period (hrs)	Culture supernatant (mL)	Observation	Control
12	100	Hides were softer, mild removal of hairs	No hairs removed
24	100	100% removal of hairs by gentle rubbing itself	No hairs removed

3.5 Partial Purification of Protease

Acetone precipitation proved to be the best method of precipitating the protease enzyme from T5 culture supernatant. The partially purified enzyme was stored carefully for further purification.

4. Discussion

Tannery lime effluent was selected for screening of proteolytic bacteria, as many alkalophilic proteolytic bacteria will be found growing in the lime effluent with its pH around 12-14. A proteolytic strain named *Bacillus pumilus* was isolated and checked for its dehairing activity. It showed considerably good results of complete unhairing of hides within 24 hours of incubation, which was comparatively less efficient than that of *Ekhlas et al.*, 2014 [1]. Protease enzyme from *Bacillus pumilus* supernatant was partially purified with acetone and stored for complete purification. The above mentioned organism has to be further checked for its thermo alkaline protease production, as *Bacillus pumilus* generally has the ability of growing in high pH, salt and thermal conditions.

5. Conclusion

Chemical dehairing of raw hides leads to severe alkaline and toxic conditions in the leather industry and it is the major contributor of tannery chemical load. Enzymatic unhairing will significantly reduce this huge burden of the tanners, providing them an eco-friendly and chemical-free platform to work. Thus, microbial enzymes are gaining more significance gradually in all major economical industries, replacing their traditional methods.

6. Acknowledgment

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7. References

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