International Journal of Multidisciplinary Research and Development

Volume: 2, Issue: 10, 227-234
Oct 2015
www.allsubjectjournal.com
e-ISSN: 2349-4182
p-ISSN: 2349-5979
Impact Factor: 5.742

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Isolation and Identification of Keratinolytic Bacteria from Tannery Effluent: A Study on Their Biodegradative and Dehairing Activity

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Abstract
Degradation of keratinous wastes is a troublesome issue faced in the poultry farms (due to chicken feathers) and in tanneries (during de-hairing process). In this study, keratinolytic microorganisms were isolated from tannery aeration tank effluent. Isolates that had proteolytic activity were identified to be 6A - *Bacillus mycoides*, 8A - *Bacillus cereus*, 11A - *Bacillus vallismortis*, 12A - *Bacillus mojavensis* through MALDI-TOF. Isolate 8A (*Bacillus cereus*) showed highest degree of degradation (45%) of chicken feather when compared with other isolates and isolates 8A, 11A and 12A showed de-hairing activity. The enzymes were partially purified using acetone precipitation. Protein estimated was found to be 1.5 µg/ml, 2.2 µg/ml, 4.0 µg/ml, and 2.1 µg/ml for isolates 6A, 8A, 11A and 12A respectively. The molecular weight of the purified enzyme fractions obtained was approximately between 40 to 60 kDa. The present study reveals that it is advantageous to use microbial proteolytic enzymes for degradation of keratinous wastes, instead of commercial chemical and thermal hydrolysis methods.

Keywords: *Bacillus*, De-hairing, Enzymes, Feathers, Keratinolytic microorganisms.

1. Introduction
Keratin belongs to the family of fibrous structural proteins. They are the major structural component of feathers, raw leather hides and wool. Based on the sulphur content, they are classified as hard keratins and soft keratins. Keratinases are proteolytic enzymes in nature and was first coined by Kuhne in 1878. Two possible mechanisms by which keratinases degrade keratinous substrates are sulfitolysis and proteolysis.

Keratinolytic microorganisms arose as an essential alternative to steam pressure cooking of poultry wastes - chicken feathers that needed high thermal energy. These microorganisms are also gaining importance in leather industries during the de-hairing process of hides for conversion of raw hides into wet blue tanned leather. During de-hairing, large amount of lime and sodium sulphide is required for removal of hairs. These are the traditional chemicals being used in the tanneries during de-hairing process (also called as liming process by the tanners). This process is performed in special rotatory drums for a long period of time for complete removal of hairs from the hides. But this traditional method emits harmful gases such as hydrogen sulphide and ammonia, which is highly toxic and carcinogenic for the tanners. Enzymatic dehairing is increasingly seen as a reliable alternative to avoid the problem created by sulfide in tanneries [2, 10]. The advantages of enzymatic dehairing are reduction of sulfide content in the effluent, recovery of hair which is of good quality, and elimination of the bate in the deliming. However, this potential benefit remains unfulfilled as enzymes are more expensive than the conventional process chemicals and require careful control [9].

In this present study, an attempt has been made to isolate, and characterize keratinase producing microorganisms from tanneries and to check their enzymatic biodegradation of chicken feathers and enzymatic de-hairing activity of raw leather hides. With this aim in mind, the main objectives of this work are: i) to isolate the keratinase producing proteolytic bacteria from tannery effluent and their characterization using MALDI-TOF; ii) production of crude keratinase from proteolytic microorganisms; iii) to determine the enzymatic degradation of chicken feathers by proteolytic bacteria and their degree of degradation (DD) and to examine the direct de-hairing activity of those microorganisms and iv) partial purification of keratinase enzyme and determination of molecular weight of the enzyme.

2. Materials and Methods

2.1 Sample Collection

For the isolation and identification of keratinolytic bacteria, tannery effluent samples from aeration tank were 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 Aeration Tank Effluent

Serial Dilution was performed to enumerate the total number of viable cells present in the 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 - 48 hours. Total number of viable cells is counted using the following formula –

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

2.3 Identification of Isolates

The isolates were identified on the basis of morphological and biochemical characters [5].

2.4 Screening of Keratin Degrading 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.

2.5 MALDI-TOF Analysis of the Keratinolytic Bacteria

Strains that produced clear zones on skim milk agar were further identified using MALDI-TOF. MALDI-TOF (Matrix Assisted Laser Desorption Ionization-Time of Flight) Mass Spectrometry was performed to identify microorganisms by their unique molecular fingerprints, using an instrument named MALDI Biotyper. An isolated colony was selected and mixed with matrix and was added to MALDI-TOF project list. Software automatically generated MALDI-TOF spectrum, which was then instantly matched against reference library to give identification, with the help of the score value obtained.

2.6 Preparation of Inoculum

Inoculum was prepared [4]. 100 ml nutrient broth solution was prepared and sterilized at 121 °C for 20 min. The medium was inoculated under aseptic conditions with bacteria. The broth culture was incubated for 14 hrs on a rotary shaker (150 rpm) at 30°C and was used for inoculating the production medium.

2.7 Degradation of Chicken Feather by the Isolated Bacteria

For studying the biodegradation of keratinous material [4], the keratinous wastes (chicken feathers) were fragmented into pieces with about 1 cm long and added to the fermentation media as a sole source of carbon and nitrogen. These sources were added separately to the fermentation media at 1% w/v. The percent of keratinous waste degradation was determined.

2.8 Determination of Degree of Degradation (DD)

The residual feather was washed, dried and scaled to calculate DD [4] by using following equation-

DD (%) = (TF-RF) ×100/TF

Where, TF is the total feather and RF is the residual feather.

2.9 Crude Keratinase Production and Its Direct De-Hairing Activity

Submerged fermentation was performed by inoculating pure culture of isolate into the production medium. The organism was grown in nutrient broth at 37ºC 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 enzymes of each isolates separately to observe enzymatic de-hairing capability of the organism. Sodium chloride (1%) was added to prevent the growth of spoilage microorganisms. Nutrient broth was used as control.

2.10 Partial Purification of Keratinase

The cell free extract from fermentation broth was partially purified by acetone precipitation method [11]. Keratinase was precipitated by prechilled 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).

2.11 Estimation of Protein

The protein content of the partially purified enzymes obtained from acetone precipitation was detected [7], using Bovine Serum Albumin (BSA) as a standard. The color developed was read at 660 nm.

2.12 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was carried out [6], using 12% cross-linked polyacrylamide gel. The molecular weight of these proteins were then compared with the help of standard molecular markers (220-14 KDa) {Bio-rad-USA}.

3. Results & Discussion

In the present investigation, keratinolytic bacteria were isolated from tanneries, as they were keratin-rich sites and degradation of feathers and de-hairing of hides was studied successfully.

3.1 Isolation of Microorganisms from Aeration Tank Effluent

The microbial load of the aeration tank effluent of the tannery industry was determined by performing serial dilution and incubating the plates at 37°C. Colonies observed in each dilution were counted and expressed in terms of cfu/mL.

Table 3.1: Serial Dilution Results of Tannery Effluent

Serial No.	Dilutions	No. of colonies	cfu/mL
1.	10^{-2}	193	1.93×10^5
2.	10^{-3}	172	1.72×10^5
3.	10^{-4}	175	1.75×10^5
4.	10^{-5}	152	1.52×10^5
5.	10^{-6}	132	1.32×10^5
6.	10^{-7}	126	1.26×10^5
7.	10^{-8}	94	0.94×10^5

The microbial load was numerous, and was highest ($1.93x10^5$) at 10^{-2} dilution and decreased gradually by $0.94x10^5$ at 10^{-8} dilution. Enormous number of different bacterial colonies were observed on the nutrient agar plates, which included both pigmented as well as non-pigmented colonies, that may be pathogenic or non-pathogenic. Hence, further identification of the colonies was performed.

3.2 Identification of Isolates

Table 3.2: Morphological and Biochemical Characterization of Isolates

Details of Experiment	Observations							
	Bacterial Isolates							
	2A	3A	4A	5A	6A	8A	11A	12A
Shape of bacteria	cocci	cocci	rod	rod	rod	rod	rod	rod
Gram character	+ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve
Spore staining	-ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve
Colony Characteristics								
Growth	rapid	rapid	rapid	rapid	rapid	rapid	rapid	rapid
Shape	round	round	round	round	circular flat	circular flat	round	round
Margin	double layer	smooth	smooth	smooth	rough	undulate	undulate	smooth
Colour	light brown	white	white	pale white	pale white	creamy white	creamy white	white
Opacity	opaque	opaque	semi transparent	transparent	opaque	opaque	opaque	opaque
Biochemical Characteristics								
Methyl red reaction	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
Voges-Proskauer reaction	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve
Citrate utilization	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Gelatinase	+ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve
Starch hydrolysis	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
Caesinase	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve
Catalase	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Urease	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve
TSI	alkaline slant & bud	alkaline slant & bud	alkaline slant & bud	alkaline slant & bud	acid bud alkaline slant	alkaline slant & bud	acid bud alkaline slant	acid bud alkaline slant
Carbohydrate Fermentation								
Glucose	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
Lactose	+ve	-ve	-ve	-ve	+ve	+ve	+ve	-ve
Sucrose	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
Maltose	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve
Gas	-ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve

Based on the above results, a preliminary identification of the genera of all the 8 isolates was done. According to the Bergey's Manual of Systematic Bacteriology, isolate 2A belonged to the genera *Staphylococcus* and 3A to *Sporosarcina,* 4A to *Klebsiella,* 5A to *Aeromonas,* and isolates 6A, 8A, 11A and 12A to *Bacillus*.

3.3 Screening of Keratin Degrading Isolates

Four isolated strains were able to form clear zones on skimmed milk agar plates. Isolates – 6A, 8A, 11A and 12A were able to form clear zones on the agar plates. Hence, these 4 isolates were further subjected to MALDI-TOF analysis for identification and then was used for application studies.

3.4 MALDI-TOF Analysis of the Keratinolytic Bacteria

Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry was performed to identify the bacteria. Based upon the score value, the organisms were identified as 6A - *Bacillus mycoides*, 8A - *Bacillus cereus*, 11A - *Bacillus vallismortis*, 12A - *Bacillus mojavensis*.

Table 3.3: MALDI-TOF results of the isolates

Analyte ID	Organism (Best match)	Score value
6A	*Bacillus mycoides*	1.953
8A	*Bacillus cereus*	1.852
11A	*Bacillus vallismortis*	1.71
12A	*Bacillus mojavensis*	1.608

3.5 Degradation of Chicken Feather by the Isolated Bacteria

Inoculum was prepared and biodegradation of chicken feather was studied. Isolate 8A (*Bacillus cereus*) showed highest degree of degradation (45%) of chicken feather when compared with other isolates. Results obtained were comparatively lower than that of the degree of degradation of chicken feather obtained by Harison Masih *et al.*, 2014 (70%) [4].

Table 3.4: Bacterial strains showing feather degradation

Bacteria	Initial Weight (Feather) (g)	Final Weight (After 4 Days) (g)	Degradation (%)
Control	1	1	0
6A	1	0.95	5
8A	1	0.55	45
11A	1	0.55	25
12A	1	0.83	17

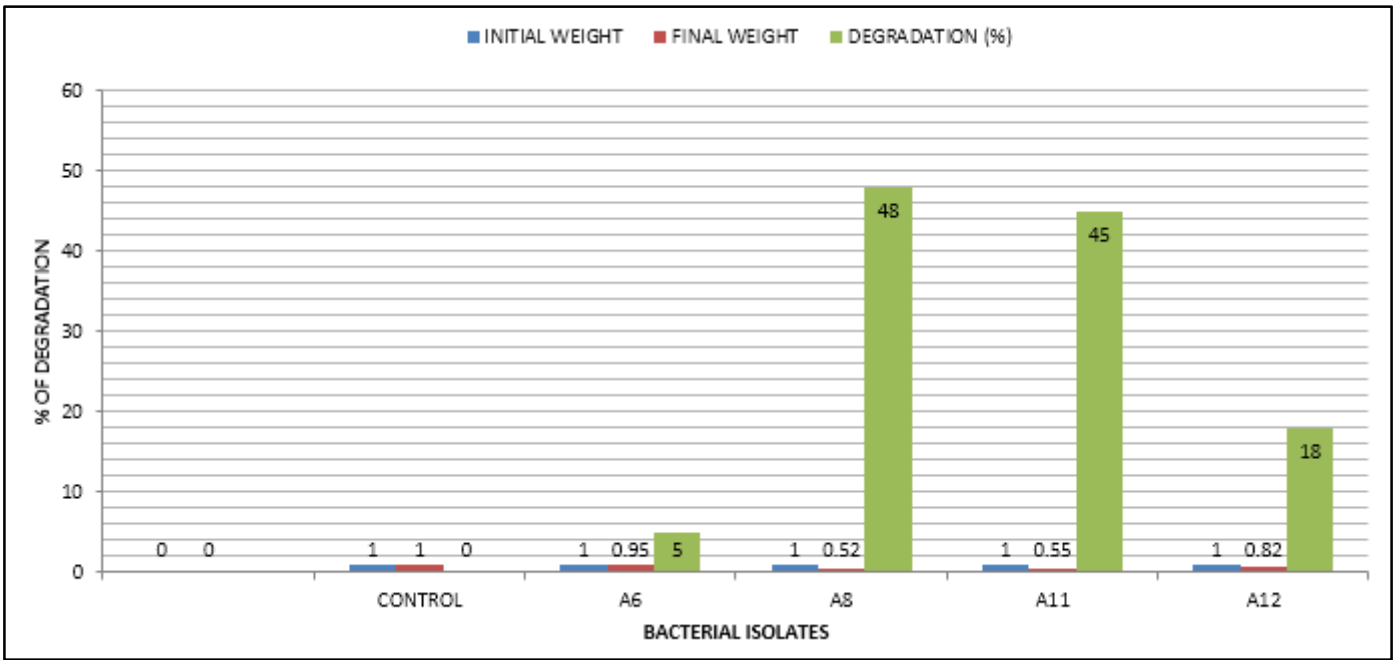

Fig 3.1: Degree of degradation (%) of chicken feather by bacterial isolates

1st day | 4th day | Dried Feather

Fig 3.2.1: Control (Without bacterial Isolates)

1st day | 4th day | Dried Feather

Fig 3.2.2: Degradation of chicken feather by *Bacillus mycoides* (6A)

1st day 4th day Dried Feather

Fig 3.2.3: Degradation of chicken feather by *Bacillus cereus* (8A)

1st day 4th day Dried Feather

Fig 3.2.4: Degradation of chicken feather by *Bacillus vallismortis* (11A)

1st day 4th day Dried Feather

Fig 3.2.5: Degradation of chicken feather by *Bacillus mojavensis* (12A)

Fig 3.2: Degradation of chicken feather by the bacterial isolates

3.6 Direct De-Hairing Activity of the Enzymes

The hair removing activity of the culture filtrate was tested on 4″×4″ raw leather hides for different time interval. Hair removing efficiency increased with incubation time. Isolates 8A, 11A, 12A showed de-hairing activity due to their ability to degrade the keratins present on the leather hides but isolate 6A did not show de-hairing activity. Removal of hairs was observed by gentle rubbing itself after 24 hours of incubation.

Table 3.5: Direct De-Hairing Activity of the Enzymes

Incubation period (hours)	Culture growth period (hours)	Culture supernatant (ml)	Observation				Control
			Enzyme Effects Of Bacteria Sample				
			6A	8A	11A	12A	
24	48	100	No hair removed from the skin.	Most of the hairs were removed by gentle rubbing.	Most of the hairs were removed by gentle rubbing.	Most of the hairs were removed by gentle rubbing.	No hair removed
24	72	100	No hair removed from the skin.	100% hair removal obtained by gentle rubbing.	100% hair removal obtained by gentle rubbing.	Most of the hairs were removed by gentle rubbing.	

Control 48 hrs Culture 72 hrs Culture

Fig 3.3.1: Direct De-Hairing Activity of the enzyme isolated from *Bacillus mycoides* (6A)

Control 48 hrs Culture 72 hrs Culture

Fig 3.3.2: Direct De-Hairing Activity of the enzyme isolated from *Bacillus cereus* (8A)

Control 48 hrs Culture 72 hrs Culture

Fig 3.3.3: Direct De-Hairing Activity of the enzyme isolated from *Bacillus vallismortis* (11A)

Control 48 hrs Culture 72 hrs Culture

Fig 3.3.4: Direct De-Hairing Activity of the enzyme isolated from *Bacillus mojavensis* (12A)

Fig 3.3: Direct De-Hairing Activity of the Bacterial Enzymes

3.7 Partial Purification of Keratinase and Estimation of Protein

The enzymes were partially purified using acetone precipitation. Protein estimation of the partially purified enzymes was carried out by Folin – Lowry Method using BSA as a standard and found to be 1.5 µg/ml, 2.2 µg/ml, 4.0 µg/ml, and 2.1 µg/ml for isolates 6A, 8A, 11A and 12A respectively.

3.8 SDS-PAGE

After the purification of enzyme was done by using acetone precipitation, the purified enzymes were collected for the analysis of their molecular weight using SDS-PAGE. These fractions were loaded on to SDS-PAGE set up. The molecular weight of the purified fractions was determined on comparison with the standard molecular weight markers. The apparent molecular weight of keratinase was found to be 50 kDa. The molecular weight of the purified enzyme fractions obtained was approximately between 40 to 60 kDa.

4. Conclusion

From the above results and discussion, it may be concluded and suggested that the microbial proteolytic enzymes could be used as an efficient alternative to steam cooking of feathers for their degradation. And satisfactory results of de-hairing suggested that these enzymes could be used for de-hairing process in tanneries to control the pollution load caused by the chemicals. This will simultaneously eliminate the health disorders caused to the public as well as the people working in the poultry farms and tanneries.

5. Acknowledgment

The authors are thankful to E.K.M Leather Processing Company, Erode, Tamil Nadu, India for their gratis supply of effluent from aeration tank and raw leather hides.

6. References

1. Adriano Brandelli. Bacterial Keratinases: Useful Enzymes for Bioprocessing Agroindustrial Wastes and Beyond. *Food Bioprocess Technology* 2007; 1: 105-116.

2. Cantera, C. S. Hair saving unhairing process. Remarks on the evolution of the investigation on enzyme unhairing. *Journal of the Society of Leather Technology Chemists* 2001; 85: 836– 841.
3. Ekhlas Uddin, Pulak Maitra, Hossain, Faruquee and Firoz Alam. Isolation and characterization of proteases enzyme from locally isolated bacillus sp. *American Journal of Life Sciences* 2014; 2(6): 338-344.
4. Harison Masih and Sandeep Singh. Degradation of Keratinous Waste Products by Keratinolytic Bacteria Isolated from soil. *International Journal of Engineering and Computer Science* 2014; 3: 7588-7595.
5. Holt J.G, D. H. Bergey, N.R Krieg. Bergey's Manual of systematic bacteriology. Vol Williams and Wilkins, 1984. Baltimore, USA.
6. Laemmli U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227(5259): 680-685.
7. Lowry, O.H, Rosebrough, N.J, Farr, A.L, and Randall, R.J. *J.Biol.Chem* 1951; 193: 265.
8. Manju, R. Isolation, Identification, Characterization of Bacillus subtilis producing the Keratinase Enzyme under Optimization, Purification and immobilization method. *International Journal of Advanced Research* 2013; 1: 456-465.
9. Schraeder, C. E, Ervin, R. T. and Eberrspacher, J. L. Economic analysis of the feasibility of using enzymes in the unhairing process. *Journal of the American Leather Chemists Association* 1998; 93: 265–271.
10. Thanikaivelan, P, Rao, J. R, Nair, B. U. and Ramasami, T. Progress and recent trends in biotechnological methods for leather processing. *Trends in Biotechnology* 2004; 22: 181–188.
11. Venkata Saibabu., Francois Niyongabo Niyonzima. and Sunil S. More. Isolation, Partial purification and Characterization of Keratinase from Bacillus megaterium. *International Research Journal of Biological Sciences* 2013; 2(2): 13-20.