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A comparative study on alkaline protease production from *Bacillus spp*. and their biodegradative, dehairing and destaining activity

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Abstract
Proteolytic *Bacillus* strains isolated from tannery effluent were screened on saline skim milk agar plates (pH-9) for their potency to secrete alkaline protease. *Bacillus licheniformis* exhibited highest extracellular alkaline protease activity of 360 Units/ml at 72hrs of incubation. Among the proteolytic isolates, *Bacillus licheniformis* degraded chicken feathers to the maximum extent (58%), followed by *Bacillus cereus* M-1 (40%) and *Bacillus subtilis* (37%). Dehairing activity of alkaline protease revealed that they were efficient when employed in dip method, than spray and paint method. Complete destaining was observed within 20mins by the crude enzyme produced from *Bacillus licheniformis.* Crude enzymes were partially purified by acetone precipitation and SDS-PAGE results revealed that the molecular weight of the alkaline protease enzyme was 28kDa.

Keywords: Alkaline protease, *Bacillus*, Dehairing, Destaining, Feather

1. Introduction
Microbial enzymes are potentially useful in a broad range of industrial and agricultural sectors. Proteases hold a major share of the global enzyme market due to their wide range of applications and availability. Alkaline proteases constitute a major role in tanneries and detergent industries as they are stable and capable of overcoming extreme environmental conditions [1]. Keratin is an insoluble fibrous protein found in hair, wool, feather, nail and horn which is highly rigid, made up of beta-helical sheets linked through cysteine bridges. Poultry feathers have high mechanical stability and resistance to proteolytic degradation because of the presence of disulphide, hydrogen bonds and salt linkages. Current treatment methods for degrading feathers involve thermal hydrolysis at high temperatures and pressure. By doing so, essential amino acids are ruptured and can no longer be used. Need for an alternative hydrolysis method has led to the utilization of microbial enzymes (proteases) for degrade keratinous substrates into simpler usable forms.

Proteases with high keratinolytic activity and low collagenolytic activity are selectively used in de-hairing process of leather industries. As an alternative to sodium sulphide and lime, enzymatic dehairing is an emerging technology for safe removal of hairs from hides, as toxic chemicals produce obnoxious odor and elevate COD levels of effluents, which is extremely hazardous to the manpower. Alkaline proteases are widely used in detergent industries for removal of proteinaceous stains. They are added as detergent additive for their efficiency in making cloth washes reliable and time consuming. Thus the current work aims at screening alkaline protease producers from tannery effluent and evaluating their activity. Three potential applications of alkaline protease have been tried and discussed – feather degrading activity, dehairing activity and destaining activity.

2. Materials and Methods
2.1 Microorganisms and Chemicals
Bacillus subtilis, *Bacillus cereus* M-1, *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus mycoides*, *Bacillus cereus* A-1, *Bacillus vallismortis* and *Bacillus mojavensis* employed in this research work were previously isolated from tannery effluent (pH 12) and maintained on nutrient agar slants in our laboratory (2015) until use. Feathers used for degradation studies were collected from a slaughter house located in Erode district of Tamil Nadu and raw hides were procured from E.K.M. Leather Processing Company, Erode District of Tamil Nadu, India. All the media components were of analytical grade and solvents were purchased from Merck.

2.2 Screening for protease production
Screening of proteolytic bacteria was carried out [2]. The bacteria were inoculated (pin-point inoculation) on skim milk agar plates containing 2.5% (w/v) skimmed milk, 0.5% (w/v) peptone, 1.0% (w/v) NaCl and 1.5% (w/v) agar, to test their proteolytic activity and incubated at 37°C for 24 h. Strains that formed transparent zones, due to hydrolysis of milk casein, after 24h of incubation were taken as evidence for protease production.

2.3 Screening for alkaline protease production
Alkaline protease producing bacteria were screened [2]. One hundred µl of overnight grown broth culture of each protease producing isolates were loaded in the wells created aseptically on saline milk agar plates (pH-9) and the plates were then incubated at 37ºC for 24 – 36h. The isolates having maximum clearance zone were selected. The alkaline protease producing bacterial colonies were grown (37°C) and maintained on nutrient agar slants at 5°C until use.

2.4 Production of crude alkaline protease enzyme

The selected pure culture of each proteolytic isolate was inoculated into 100 mL of alkaline protease broth (0.5% glucose, 0.75% peptone, 0.25% yeast extract, 0.1% K_2HPO_4, 0.1% $MgSO_4$, 1.0% Na_2CO_3, 1.0% NaCl), pH of the medium was adjusted to 9.0 and incubated at 37°C [2]. The samples were withdrawn aseptically at regular intervals of every 24 hour. The fermented broth was centrifuged at 10,000 rpm for 5mins. The obtained cell – free supernatant was used as crude enzyme for alkaline protease assay.

2.5 Quantitative assay of extracellular alkaline protease activity

The proteolytic activity was quantitatively assayed by a slightly modified method [2]. The reaction mixture contained 1.0 ml of crude enzyme extract and 5.0 ml of 1% BSA (Bovine Serum Albumin), which was incubated at 37°C for 20 min. The reaction was stopped by the addition of 4.0 ml of trichloroacetic acid, vortexed to ensure complete mixing and incubated further for 15 min at room temperature and then centrifuged at 10,000 rpm for 15 min. The supernatant was used to estimate the amount of free tyrosine released [3]. One unit of protease activity is defined as the amount of enzyme required to liberate 1.0 µg of tyrosine per min per ml under the standard assay conditions.

2.6 Inoculum preparation for feather degradation studies

The growth medium for feather degradation studies was prepared [4]. 50ml of alkaline protease broth was prepared, sterilized at 121°C for 20 mins and inoculated with the bacterial strains. The broth was incubated for 14 hrs at 37°C and used as inoculum.

2.7 Degradation of chicken feather

Degradation of chicken feather was carried out [4]. The feathers are cut into small pieces of about 1cm long and added (1% w/v) to the fermentation media to serve as sole carbon and nitrogen source. The resulting degradation of keratinous waste was determined in percentage (%).

2.8 Determination of Degree of Degradation (DD)

Degree of degradation was performed as per the method [4]. The residual feather was washed and dried thoroughly to evaluate degree of degradation by using the following equation:

DD (%) = (TF-RF) ×100/TF

where, TF – Total Feather and RF – Residual Feather. The fermented silage was air-dried at 50°C for 12 hrs to obtain feather powder.

2.9 Proximate Composition of the Hydrolyzed Feather Powders

As per the standard methods [5], feather powders was examined for total protein, total fat, ash, and crude fiber content and carbohydrate content was analyzed [6].

2.10 Crude Alkaline Protease Enzyme Production

Alkaline protease production was carried out through submerged fermentation for a period of 3 days [7]. The fermented broth was centrifuged at 4000 rpm for 8 minutes. The supernatant was used as crude enzyme for dehairing studies.

2.11 De-Hairing Activity of the Enzyme

Dehairing studies were carried out by adding the crude enzyme on detergent washed bovine hides by dip, spray and paint method, to check for its ability to remove hairs from the pelt as shown in Figure 1. [8].

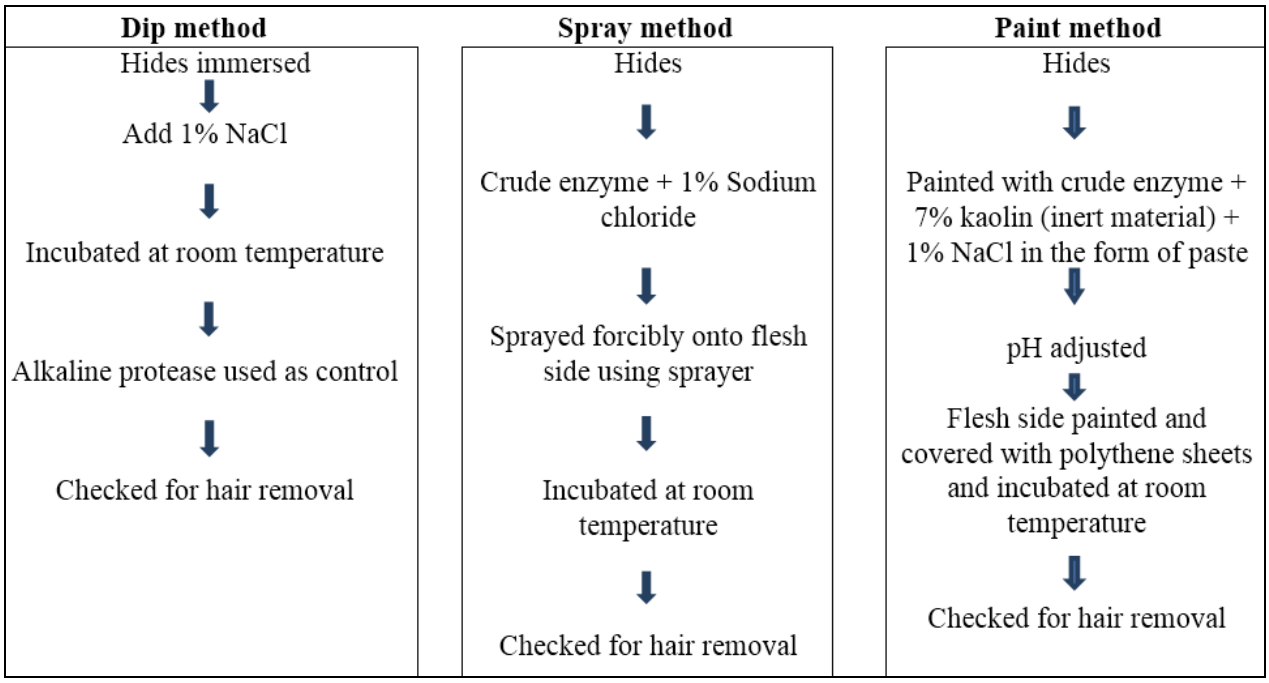

Fig 1: De-hairing Methods.

2.12 Destaining studies

Assessment of alkaline protease as a detergent additive was carried out on cotton cloth pieces (4 x 4 cm) stained with blood and spicy food, following the method [8]. The following sets were prepared.

Set I

1. Control – A: stained cloth (blood).
2. Stained cloth (blood) + distilled water (100 ml).
3. Stained cloth (blood) + distilled water (100 ml) + 1 ml detergent - 1 (5 mg/ml).
4. Stained cloth (blood) + distilled water (100 ml) + 1 ml detergent - 2 (5 mg/ml).
5. Stained cloth (blood) + distilled water (100 ml) + 1 ml detergent - 1 (5 mg/ml) + 1 ml enzyme solution.
6. Stained cloth (blood) + distilled water (100 ml) + 1 ml detergent - 2 (5 mg/ml) + 1 ml enzyme solution.
7. Stained cloth (blood) + distilled water (100 ml) + 2 ml enzyme solution.

Set II

1. Control – B: stained cloth (spicy food).
2. Stained cloth (spicy food) + distilled water (100 ml).
3. Stained cloth (spicy food) + distilled water (100 ml) + 1 ml detergent - 1 (5 mg/ml).
4. Stained cloth (spicy food) + distilled water (100 ml) + 1 ml detergent - 2 (5 mg/ml).
5. Stained cloth (spicy food) + distilled water (100 ml) + 1 ml detergent - 1 (5 mg/ml) + 1 ml enzyme solution.
6. Stained cloth (spicy food) + distilled water (100 ml) + 1 ml detergent - 2 (5 mg/ml) + 1 ml enzyme solution.
7. Stained cloth (spicy food) + distilled water (100 ml) + 2 ml enzyme solution.

2.13 Partial purification of Alkaline Protease Enzyme

Partial purification of alkaline protease enzyme was carried out by acetone precipitation method. Alkaline protease was precipitated by pre-chilled acetone fractionation. Acetone was added to cell-free extract in 3:1 ratio and was incubated for 60 mins at -20°C. The fermented broth was centrifuged at 10,000 rpm for 10 mins. The supernatant was discarded and dissolved in Tris - acetate buffer (pH 7) [9]. Protein content of partially purified enzymes was evaluated [3].

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

Molecular weight of alkaline protease enzymes were carried out according to the method by SDS-PAGE using coomassie staining [10]. The molecular weight of the protein bands were determined by comparing with the standard molecular marker (30-10 kDa) (Bio-rad-USA).

3. Results & Discussion

3.1 Screening for protease production

Among the eight *Bacillus* strains screened for proteolytic activity, three isolates (Table.1) showed maximum zone of inhibition and were selected for further fermentation studies for alkaline protease production. This may be attributed to the secretion of extracellular protease enzyme that hydrolyzed casein present in the skim milk plates.

Table 1: Zone of inhibition in skim milk agar plates.

S. No	Bacterial Strain	Zone diameter (mm) after 24 hrs incubation
1.	*Bacillus licheniformis*	35mm
2.	*Bacillus cereus* M-1	29mm
3.	*Bacillus subtilis*	23mm

3.2 Screening for alkaline protease production

The selected isolates were screened for alkaline protease enzyme production on saline skim milk agar plates (pH-9). The results of the screening studies indicated that maximum zone of clearance and thereby maximum alkaline protease activity was exhibited by *Bacillus licheniformis* followed by *Bacillus cereus* M-1 and *Bacillus subtilis* as shown in Table.2.

Table.2: Screening for alkaline protease producers.

S. No	Bacterial Strain	Zone diameter (mm)
1.	*Bacillus licheniformis*	36mm
2.	*Bacillus cereus* M-1	24mm
3.	*Bacillus subtilis*	22mm

3.3 Quantitative Assay of Extracellular Alkaline Protease

Extracellular alkaline protease activity for the selected isolates was carried out in alkaline protease production medium and the enzyme activity was measured at every 24 hr interval until a markable decrease in the enzyme activity was observed. In the present study, *Bacillus licheniformis* showed maximum alkaline protease activity (360 Units/ml) followed by *Bacillus cereus* M-1 (312 Units/ml) and *Bacillus subtilis* (309 Units/ml) at 72 hrs of incubation. The enzyme activity decreased after 72 hrs possibly due to reduction in the amount of residual nutrients for further fermentation.

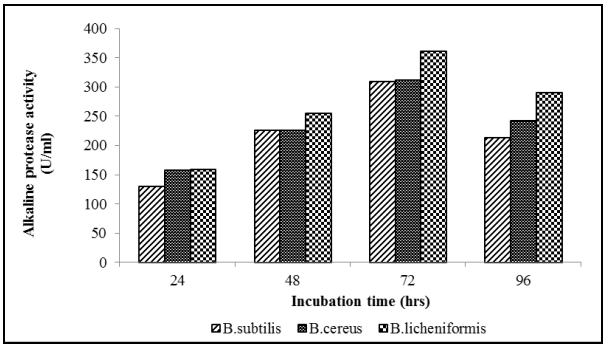

Fig 2: Graph showing alkaline protease activity (U/ml) of selected *Bacillus sp.*

3.4 Chicken feather degradation and determination of degree of degradation (DD)

The crude enzyme from *Bacillus licheniformis*, *Bacillus cereus* M-1 and *Bacillus subtilis* were used for analyzing the degree of chicken feather degradation. After 4 days of incubation, the maximum degradation capacity was exhibited by *Bacillus licheniformis* (58%) followed by *Bacillus cereus* M-1 (40%) and *Bacillus subtilis* (37%) as

depicted in Table.3. Degradation capability of the crude enzyme could be attributed to microbial keratinolysis which involves proteolysis and sulfitolysis. Later the fermented silage of each isolate was air-dried at 50 °C for 12 hrs and feather powders were obtained.

Table 3: Degree of degradation.

Organism	Initial weight of feathers (g)	Final weight of feathers (g) (After 4 days)	Degradation capacity (%)
Control	1	1	0
Bacillus licheniformis	1	0.42	58
Bacillus cereus M-1	1	0.60	40
Bacillus subtilis	1	0.63	37

3.5 Proximate Composition of the hydrolyzed feather powders

The fermented products were dried and checked for their proximate composition using standard methodologies. The proximate nutrient composition in the three fermented products was represented in Table.4. Thus, the hydrolyzed feather powders obtained were nutritious in nature and could be recommended for feed industries or bio-fertilizer applications after proper toxicological assessments.

Table 4: Proximate Composition of the hydrolyzed feather powders.

Organism	Protein (%)	Fat (%)	Amino acid (%)	Ash (%)	Crude fiber (%)
Bacillus licheniformis	6.2	9.0	2.5	1.2	1.3
Bacillus cereus M-1	5.95	11	2.4	1.9	2.3
Bacillus subtilis	5.6	10.6	2.1	2.0	1.8

Fig 3: Hydrolyzed feather products.

3.6 Dehairing activity of the crude enzymes

Comparative study of alkaline protease dehairing activity was undertaken by dip, spray and paint methods. After 24 hrs of incubation, dehairing activity was proved to be efficient in dip method, thereby highlighting the intervention of alkaline protease enzyme produced by *Bacillus licheniformis.* Satisfactory dehairing activity by dip, spray and paint method was observed in *Bacillus cereus* M-1 and *Bacillus subtilis*. The results were tabulated in Table.5.

Table 5: Dehairing activity of alkaline protease producers.

Bacillus licheniformis			***Bacillus cereus* M-1**			***Bacillus subtilis***		
Dip	Spray	Paint	Dip	Spray	Paint	Dip	Spray	Paint
Efficient	Good	Moderate	Good	Good	Moderate	Good	Good	Moderate

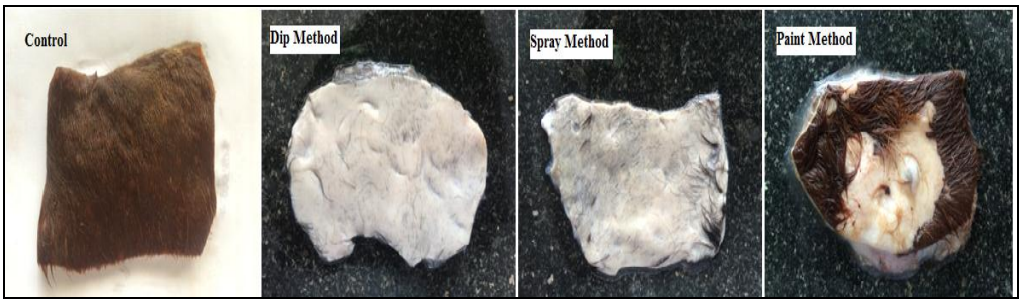

Fig 4: Dehairing activity of alkaline protease produced by *Bacillus licheniformis.*

3.7 Destaining studies

Destaining activity of alkaline protease producing strains (*Bacillus licheniformis*, *Bacillus cereus* and *Bacillus subtilis*) was compared for determining their efficiency as a detergent additive in industries. It took 25 minutes for destaining the stained clothes, when detergents alone

were used. Enzyme produced by *Bacillus licheniformis* started its destaining activity efficiently, as soon as it was added on the stained clothes, along with the detergents. Complete destaining was observed within 10 minutes of incubation, when 1 ml of the crude enzyme was added over the stained clothes, as a detergent additive. Without the addition of any detergent, crude enzyme, in higher concentration (2 ml) was checked for its destaining activity. Complete destaining was observed within 20 minutes of incubation, as the enzyme alone acted as a stain remover. The alkaline protease produced from *Bacillus licheniformis*, was compatible with both the commercial detergents used in the study, enhancing their activity and removing proteinaceous materials from the stain. Next to *Bacillus licheniformis*, enzyme produced by *Bacillus cereus* M-1 was efficient, followed by *Bacillus subtilis*.

Fig 5 A: Destaining activity of alkaline protease produced by *Bacillus licheniformis*.

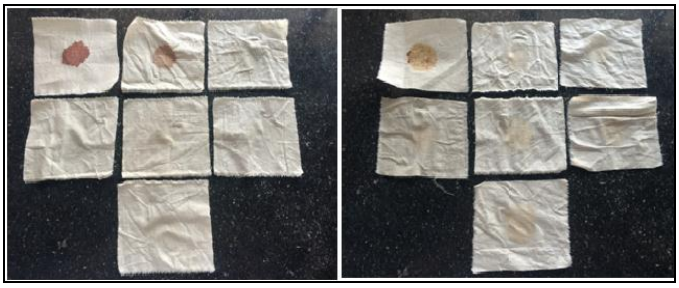

Fig 5 B: Destaining activity of alkaline protease produced by *Bacillus cereus* M-1.

Fig 5 C: Destaining activity of alkaline protease produced by *Bacillus subtilis.*

3.8 Partial Purification of Alkaline Protease Enzyme and Estimation of Protein

Total protein content as determined by Lowry's method [3] showed maximum protein content of 26 mg/ml in *Bacillus licheniformis* alkaline protease, followed by *Bacillus cereus* M-1 (24 mg/ml) and *Bacillus subtilis* (23 mg/ml).

3.9 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS – PAGE)

Molecular weight of partially purified enzymes was determined by SDS-PAGE. The results showed the presence of multiple bands indicating the partially purified nature of the enzymes. Molecular weight of the enzymes produced by the three isolates was 27kDa, 28kDa and 27kDa for *Bacillus licheniformis, Bacillus cereus* M-1 and *Bacillus subtilis*, respectively. It has been reported that the molecular weight of most of the alkaline proteases from *Bacillus sp*. lies between 16 and 32kDa [11].

4. Conclusion

The paper concludes that the alkaline protease producing *Bacillus* strains investigated in this study could be used beneficially in leather and detergent industries for their eco-friendly hair removal and stain removal properties. Alkaline protease producers are able to secrete enzymes even at harsh environmental conditions prevailing in industries. Partially purified enzymes need to be further purified and their utilization has to be implemented to replace toxic chemicals being used traditionally in many industries for the welfare of workers and environment.

5. Acknowledgment

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