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Short Communication

Isolation and Screening of Thermostable Extracellular Alkaline Protease Producing Bacteria from Tannery Effluents

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Abstract

The aim of the current study was to isolate extracellular protease producing bacteria from tannery effluents. The isolation was performed by serial dilution and plating method. Eight protease producing isolates were screened out on the basis of their clear zone formation on skim milk agar as well as production of protease in protease producing broth. They were identified on the basis of cultural, morphological and biochemical tests. Among the eight isolates, 3 isolates were found to belong to the genus *Halobacterium*, and the rest 5 isolates were found to belong to genus *Actinobacillus*. Among the eight protease producing isolates, one of the isolates was selected as promising protease producer and designated as *Halobacterium* sp. AF1.

Keywords: *Actinobacillus*; Detergent; *Halobacterium*; Protease; Proteolytic activity; Tannery.

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1. Introduction

Proteases are complex group of enzymes collectively known as peptidyl-peptide hydrolases and are responsible for hydrolysis of peptide bonds in a protein molecule [1]. Proteases are one of the most important groups of industrial enzymes accounting for more than 60% of the total enzyme sales [2]. The applications of proteases include in leather, detergent, food, meat tenderization industries. Besides that, they are also used in pharmaceuticals, medical diagnosis, and decomposition of gelatin on X-ray films as well as in textiles [3-5].

There are nearly 300 tanneries in Hazaribagh, Dhaka. Daily discharges of wastes from these tanneries are about 18,000 litres of liquid waste, 115 tones solid wastes during off-peak time. It is reported that, about 60,000 tons of raw hides and skins are processed in these tanneries every year, a process which releases nearly 95,000 litres of untreated effluents into the open environment daily [6].

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Tannery is a heavily polluted industry and is called red category industry [7]. Leather industry contributes to one of the major industrial pollution problems facing the country, and the pollution causing chemicals, viz. lime, sodium sulphide, salt, solvents, etc. arise mainly from the pre-tanning processes of leather processing [8, 9]. An important enzyme used in pre-tanning processes belongs to the group of proteolytic enzymes, proteases. For environmental reasons, the biotreatment of leather using an enzymatic approach is preferable as it offers several advantages, e.g., easy control, speed and waste reduction, thus being eco-friendly [10. 11].

Looking into the depth of microbial diversity, there is always a chance of finding microorganisms producing novel enzymes with better properties and suitable for commercial exploitation. The multitude of physicochemically diverse habitats has challenged nature to develop equally numerous molecular adaptations in the microbial world. Microbial diversity is a major resource for biotechnological products and processes [2,12]. The aim of the present study was to search thermo stable, extracellular alkaline protease producing bacteria from tannery effluents.

2. Materials and Methods

2.1. *Sampling area and sample collection*

The protein rich tannery effluent samples were collected from combined effluent of tannery industries at Hazaribagh area in Dhaka city, which is situated on the south-western part of Dhaka city. For collection of tannery effluent, two different sites of Hazaribagh Thana were selected. Duplicate sample has been collected from each site in April, 2010. Soil and water sample were collected in sterile bottle and transported to the laboratory maintaining temperature around 4˚C as early as possible for microbiological analysis.

2.2. *Isolation and screening of extracellular protease producing bacteria*

Ten gram/ml of air-dried soil sample / water samples were added to 90 ml of sterile water in an Erlenmeyer flask. The flask was then heated in a water bath for 15 min at 70 °C while the contents were agitated. Subsequently, 1 ml of the suspension was added to 9 ml of sterile distilled water and a serial dilution (10^{-1} to 10^{-6}) was prepared. One ml of each dilution was added and distributed onto skim milk agar plates. Plates were incubated at 50 °C for 24-72 h. Formation of halo zone around the colonies, resulting from casein hydrolysis, was taken as evidence of proteolytic activity. Among the clear zone forming colonies, only larger zone forming colonies were selected for purification and further study.

2.3. *Purification of the isolates*

The isolated organisms were purified through repeated subculture method. Streak plate methods were used for this purpose. Nutrient agar was used as media. When a plate

yielded only one type of colony, the organisms were considered to be pure. The purification of the isolates was also confirmed by microscopic observation.

2.4. *Identification of the bacterial isolates*

Different morphological and biochemical characteristics accompanied with colony characteristics were observed for the identification of bacterial isolates.

2.5. *Cultural condition for the production of alkaline protease*

A loopful of culture from agar plate was inoculated into 100 ml-glass tube containing 10 ml of alkaline protease production medium, and incubated overnight at 180 rpm and 50 °C. Five ml of this culture was then inoculated into 500 ml capacity Erlenmeyer flask containing 95 ml of the same medium and incubated at 50 °C in an orbital shaker for 72 h. Cells and insoluble materials were removed by centrifugation at 10 000 g for 10 min at 4 °C and the cell free supernatant was filtered through a 0.2-µm pore-size membrane filter and was used as the source of crude enzyme.

2.6. *Alkaline protease assay*

Protease activity was determined by a slightly modified method of Yang and Huang [13]. The reaction mixture containing 2 ml of 1 % casein solution in 50 mM glycine-NaOH buffer (pH 11) and 1 ml of enzyme solution were incubated at 60 °C for 15 min and the reaction was then stopped or terminated with the addition of 3 ml of 10 % trichloroacetic acid (TCA). After 10 min the entire mixture was centrifuged at 10 000 g for 10 min at 4 °C and the absorbance of the liberated tyrosine was measured with respect to the blank (non-incubated sample) at 280 nm. One protease unit was defined as the amount of enzyme that releases 1 µg of tyrosine per ml per minute under the defined assay conditions. Standard curve of tyrosine was done using 1, 2, 4, 8, and 16 µg/ml tyrosine in 50 mM glycine-NaOH buffer, pH 11.

2.7. *Total protein determination*

Protein content of the enzyme solution was measured by the method of Lowry *et al*. [14] using bovine serum albumin (BSA) as standard.

3. Results and Discussion

3.1. *Isolation and screening of thermo-stable extracellular alkaline protease producing bacteria*

For the isolation of thermo-stable extracellular alkaline protease producing bacteria, soil and effluent samples were collected from Hazaribagh tannery area. Screening of protease producing bacteria was performed using skim milk agar media. Clear zone forming

colonies were sub-cultured for the purification of the isolates (Figure 1). By visual observation, bigger clear zone forming eight isolates were selected. Clear zones were formed because of the hydrolysis of casein by protease produced from the isolates. Since the fat content of the whole milk inhibits the growth of bacteria, skim milk was used throughout the present study. Vidyasagar *et al*. [15] have used skimmed milk for the production haloalkaline proteases from *Halogeometricum* sp TSS101. Repeated subculture were performed up to uniform colonies were found. Finally, the eight (8) isolates were stocked in nutrient agar slant and stored at 4˚C for further work. Such type of method was used by Srinivasan *et al*. [16] for the isolation of thermostable protease producing bacteria from tannery effluent.

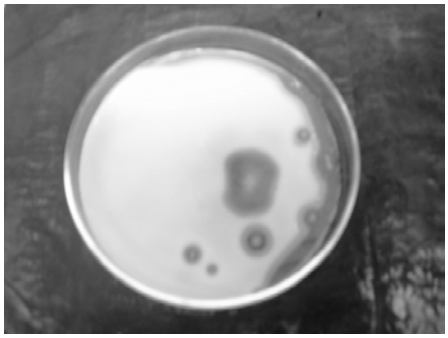

Fig. 1. Clear zone forming colonies at 10^{-4} fold diluted soil sample.

3.2. *Identification of thermostable extracellular alkaline protease producing bacteria*

In this study, colony characteristics, morphological characteristics and various biochemical tests are performed for identification of these thermo-stable extracellular alkaline protease producing isolates. Colony characteristics of these 8 isolates were observed after growing on nutrient agar plate after 48 h at 37˚C. Bacterial identification was completed performing cultural, morphological and biochemical tests and the results of these tests were correlated with the characteristics of bacteria described in Bergey's manual of determinative bacteriology [17].

3.3. *Colony characteristics of isolates*

Identification of bacteria was traditionally performed by isolating the organism and studying it phenotypically by means of gram staining, culture and biochemical methods, which were once the gold standard of bacterial identification [18]. The colony characteristics of these 8 isolates are shown in Table 1. Most of these isolates were creamy white in colour, rhizoid shape with regular to irregular edge. The texture of these isolates was mucoid and was flat elevated.

Table 1. Colony characteristics of thermostable extracellular alkaline protease producing isolates.

Colony characteristics	Isolates							
	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Isolate 6	Isolate 7	Isolate 8
Colour	Creamy white	Creamy white	Creamy white	Creamy white	Creamy white	Creamy white	Creamy white	Creamy white
Shape	Rhizoid	Rhizoid	Rhizoid	Rhizoid	Rhizoid	Rhizoid	Rhizoid	Rhizoid
Edge	Regular	Regular	Regular	Irregular	Irregular	Irregular	Irregular	Irregular
Elevation	Flat	Flat	Flat	Flat	Flat	Flat	Flat	Flat
Texture	Mucoid	Mucoid	Mucoid	Mucoid	Mucoid	Mucoid	Mucoid	Mucoid

3.4. *Morphological and biochemical characteristics of the isolates*

The results of morphological and biochemical tests are shown in Table 2 and 3. First 3 of the 8 isolates could not retain the stain of crystal violet because of the thin peptidoglycan layer in the periplasm of these bacterial isolates. The members of *Halobacterium* genus have irregular rod-shaped cells staining Gram negative (Table 2) and most of the species of this genus, including *Halobacterium salinarium*, are motile [17]. They

Table 2. Morphological and biochemical characteristics of the isolates.

Isolates	Gram reaction		Cetrimide	Oxidase	Catalase	MR	VP	Indole	Starch hydrolysis	Proteolysis	Citrate	Growth in 0.5% NaCl	Gelatin liquefaction
	+/-	Shape											
Iso-1	-	rod	-	+	+	-	-	-	+	+	-	+	+
Iso-2	-	rod	-	+	+	-	-	-	+	+	-	+	+
Iso-3	-	rod	-	+	+	-	-	-	+	+	-	+	+
Iso-4	-	rod	-	+	+	-	-	-	+	+	-	+	+
Iso-5	-	rod	-	+	+	+	-	-	+	+	-	+	+
Iso-6	-	rod	-	-	+	-	-	-	+	+	-	+	+
Iso-7	-	rod	-	+	+	+	-	-	+	+	-	+	+
Iso-8	-	rod	-	-	+	-	-	-	+	+	-	+	+

MR: Methyl Red; VP: Voges-Proskauer

produced free oxygen as gas bubbles in catalase reaction and showed oxidase positive reaction by forming indophenols blue colour. These three isolates hydrolyse the starch, gelatin, and urea by producing amylase, gelatinase and urease. These isolates showed negative results in methyl red, voges-proskauer, and indole formation tests. These isolates were unable to grow on citrate agar. These isolates also showed negative results in carbohydrate fermentation tests and H_2S production. The first three of these isolates were motile (Table 3).

Table 3. Biochemical characterization of the isolates.

Isolates	KIA			LIA			MIU		
	Glucose	lactose	H_2S	Deamination	Decarboxylation	H_2S	Motility	Indole	Urease
Iso-1	-	+	-	+	-	-	+	-	+
Iso-2	-	-	-	-	+	-	+	-	+
Iso-3	-	+	-	-	+	-	+	-	+
Iso-4	-	+	-	-	-	-	-	-	-
Iso-5	+	+	-	+	-	-	-	-	+
Iso-6	-	-	-	+	-	-	-	-	+
Iso-7	+	+	-	-	-	-	-	-	+
Iso-8	-	+	-	-	-	-	-	-	+

Table 3 (contd.)

Isolates	Carbohydrate fermentation			
	Arabinose	Glucose	Sucrose	Glycerol
Iso-1	-	-	-	-
Iso-2	-	-	-	-
Iso-3	-	-	-	-
Iso-4	-	-	-	-
Iso-5	-	-	-	-
Iso-6	-	-	-	-
Iso-7	-	-	-	-
Iso-8	-	-	-	-

KIA : Kligler's Iron Agar; LIA: Lysine Iron Agar; MIU: Motility Indole Urease.

According to Buchanan and Gibbons [17] amino acids are required for the growth of *Halobacterium* and carbohydrates are not utilized by these bacteria. *Halobacterium* are strongly proteolytic and found in highly saline environment. By comparing the results from Table 1, 2, and 3 with Bergey's manual of determinative bacteriology, it can be concluded that the first 3 isolates were *Halobacterium* sp. They were designated as *Halobacterium* sp. AF1, *Halobacterium* sp. AF2 and *Halobacterium* sp. AF3. There are many documents on the production of protease by *Halobacterium*. Capiralla *et al*. [19] has reported a hydrophobic amino acid specific endopeptidase from *Halobacterium halobium* S9 with potential application in debittering of protein hydrolysates. Many of the extracellular proteases produced by haloarchaea are likely to generate oligopeptide, dipeptide and amino acid intermediates, which feed into central metabolism. Three different haloarchaeal genera: *Halobacterium, Haloarcula and Haloferax* from salt pans of Kandla and Bhayander were isolated for protease production [20].

The rest 5 Gram negative rod shaped isolates were non-motile and catalase positive. Some of them were oxidase positive organisms. These showed positive results for urease and negative results for indole, MR, VP and Lysine decarboxylation. The colonies of these isolates were so sticky and it was difficult to remove them from bacterial agar plate. Results for the rest 5 isolates in Table 1, 2 and 3 were coincided with the characteristics of *Actinobacillus* described in Bergey's manual of determinative bacteriology. So, it can be concluded that the rest 5 isolates were *Actinobacillus* sp. and they were designated as *Actinobacillus* sp. AF4, *Actinobacillus* sp. AF5, *Actinobacillus* sp. AF6, *Actinobacillus* sp. AF7 and *Actinobacillus* sp. AF8. There are some documents on the production of alkaline

protease by *Actinobacillus*. According to Negrete-Abascal *et al*. [21] Actinobacillus *suis* secretes metalloproteases into its medium.

3.5. *Confirmation of potential thermo-stable extracellular alkaline protease producing isolates*

Eight isolates were tested for their ability of extracellular alkaline protease production with respect to their proteolytic activity. All these selected isolates were aerobically cultivated in skim milk broth for the production of extracellular alkaline protease. The organisms were inoculated in skim milk media and incubated in orbital shaker at 180 rpm for 72 h. The shaker temperature was set at 40°C for this purpose. The proteolytic activity of these 8 isolates is shown in Fig. 2. From these 8 isolates, protease produced by isolate 1 showed higher proteolytic activity than that of other isolates. In Fig. 2 corresponding media protein are also shown.

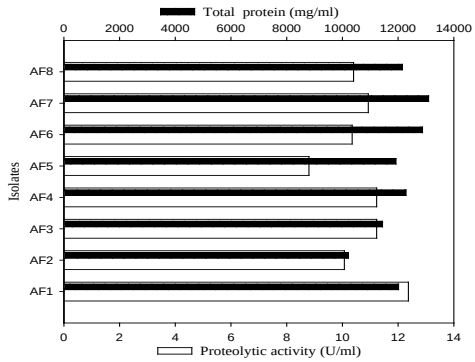

Fig. 2. Proteolytic activity of 8 different isolates with corresponding protein in protease production media.

From 3 isolates of *Halobacterium* sp. AF1 showed higher proteolytic activity than that of others (Fig. 2). Extracellular proteases isolated from Haloarchaea are mainly serine type proteases and most appear closely related to the subtilisin-like serine protease subfamily S8A (COG1404) retaining the conserved 'Ser-His-Asp' catalytic triad and was denoted as halolysins. Halolysins was identified in *Natrialba asiatica* 172P1 [22], *Haloferax mediterranei* R4 [23,24] and *Haloferax mediterranei* VKMB-1538 [25]. From 5 isolates of *Actinobacillus*, *Actinobacillus* AF4 showed higher proteolytic activity than others did. Wang *et al.* [26] showed that the protease from *Actinobacillus actinomycetemcomitans* revealed trypsin-like activity with hydrolytic activity for the synthetic substrates and the activity of the protease was stable at pH 7.0 to about 8.0.

4. Conclusions

From the tannery effluent eight thermo-stable extracellular alkaline protease producing bacteria were isolated on the basis of formation of clear zone in skim milk agar plates. Three of the isolates were found to belong to the genus *Halobacterium* and five isolates

were found to belong to genus *Actinobacillus*. Among the isolates, *Halobacterium* sp. AF1 showed higher proteolytic activity (12.4 U/ml), compare to that of other isolates.

References

1. R. Gupta, B. Chauhan, P. Ramnani, and R. Singh, Microbial Diversity: Current Perspectives and Potential Applications, T. Satyanarayana and B.N. Johri (ed.) (I.K. International, New Delhi, 2005) pp. 769-789.
2. R. Gupta, Q. K. Beg, and P. Lorenz, Appl. Microbiol. Biotechnol. **59,** 15 (2002). http://dx.doi.org/10.1007/s00253-002-0975-y
3. H. S. Joo, C. G. Kumar, G. C. Park, K. M. Kim, S. R. Paik, and C. S.Chang, Process Biochem. **38**, 155 (2002). http://dx.doi.org/10.1016/S0032-9592(02)00061-4
4. R. Patel, M. Dodia, and S. P. Singh, Process Biochem. **40**, 3569 (2005). http://dx.doi.org/10.1016/j.procbio.2005.03.049
5. C. Tari, H. Genckal, and F. Tokatali, Process Biochem. **41**, 659 (2006). http://dx.doi.org/10.1016/j.procbio.2005.08.012
6. M. G. Rusal, I. Faisal, and M. M. K. Khan, International Journal of Environment and Pollution, **28,** 44 (2006).
7. M. Farooque and H. S. Rizwana, Laws Regulating Environment in Bangladesh. Bangladesh Environmental Lawyers Association (BELA, 2004). P-850. ISBN-984-8173-04-8.
8. UNEP/IEO, Tanneries and the environment - a technical guide, Tech. report series no. 4 (1991).
9. R. Agrawal, R. Singh, A. Verma, P. Panwar, and A. K. Verma. World J. Agric. Sci. **8,** 129 (2012).
10. L. P. Andersen, Method for dehairing of hides or skins by means of enzymes. US Patent. **5**, 834, 299 (1998).
11. A. Verma, H. S. Pal, R. Singh, and S. Agarwal. Int. Jr. of Agril. Env. Biotech. **4**, 173 (2011)
12. S Shankar. Chapter 3, Introduction, Ph.D. Thesis.University of Pune, India. (2010)
13. S. S. Yang and C. I. Huang, J. Chinese Agri. Chem. Soc. **32,** 589 (1994).
14. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, J. Bio. Chem. **193**, 265 (1951).
15. M. Vidyasagar, S. Prakash, C. Litchfield, and K. Sreeramulu, Archaea. **2**, 51 (2006). http://dx.doi.org/10.1155/2006/430763
16. T. R. Srinivasan, S. Das, V. Balakrishnan, R. Philip, and N Kannan, Recent Res. Sci. Technol. **1**, 63 (2009).
17. R. F. Buchanan and N. F. Gibbons, Bergey's Manual of Determinative Bacteriology, 9th edn., (Williums and Wilkins Co., Baltimore, USA, 1994).
18. P. C. Y. Woo, K. W. Leung, and K. Y. Yuen, J. Clin. Pathol. Mole. Pathol. **53**, 211 (2000). http://dx.doi.org/10.1136/mp.53.4.211
19. H. Capiralla, T. Hiroi, T. Hirokawa, and S. Maeda, Process Biochem. **38**, 571 (2002). http://dx.doi.org/10.1016/S0032-9592(02)00180-2
20. A. V. Akolkar, G. M. Deshpande, K. N. Raval, D. Durai, A. S. Nerurkar, and A. J. Desai, J. Basic Microbiol. **48**, 1 (2008). http://dx.doi.org/10.1002/jobm.200800012
21. E. Negrete-Abascal, S. V. Pacheco, G. L. Paniagua, A. P. Me´ndez, J. I. Caballero, V. M. Pe´rez Ma´rquez, and V. R. Tenorio, Cur. Microbiol. **49**, 55 (2004). http://dx.doi.org/10.1007/s00284-004-4279-6
22. M. Kamekura, Y. Seno, M. L. Holmes, and M. L. Dyall-Smith, J. Bacteriol. **174**, 736 (1992).
23. M. Kamekura and Y. Seno, Experientia **49,** 503 (1993). http://dx.doi.org/10.1007/BF01955152
24. M. Kamekura, Y. Seno, and M. Dyall-Smith, Biochem Biophys Acta. **1294**, 159 (1996). http://dx.doi.org/10.1016/0167-4838(96)00016-7
25. V. M. Stepanov, G. N. Rudenskaya, L. P. Revina, Y. B. Gryaznova, E. N. Lysogorskaya, I. Y. Filippova, and I. I. Ivanova, Biochem. J. **285**, 281 (1992).
26. P. L. Wang, S. Shirasu, M. Shinohara, M. Daito, T. Fujii, Y. Kowashi, and K. Ohura, European J. Oral Sci. **107,** 147 (1999). http://dx.doi.org/10.1046/j.0909-8836.1999.eos107211.x