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PROTEOLYTIC *BACILLUS* SPP. ASSOCIATED WITH TANNERY INDUSTRIES: CONVENTIONAL AND MOLECULAR IDENTIFICATION

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Key words: Bacteria, *Bacillus*, Tannery, Protease, Identification

Abstract

Samples collected from different stages of the tannery industry were found to be alkaline (pH 7.52 to 12.11). A good number of bacteria were found to be associated with the samples. The bacterial count ranged in between 1.34×10^5 to 3.44×10^5 cfu/ml and 1.04×10^5 to 6×10^5 cfu/ml on nutrient agar (NA) and peptone yeast extract glucose agar (PYG) medium, respectively. The maximum bacterial count was observed in bating stage while the minimum count was in the deliming stage. Primarily, 70 isolates were selected based on their different colonial morphology. After heat shock test 27 isolates were finally selected for identification and proteolytic potential. All the selected isolates were the members of different species of the genus *Bacillus*. The conventionally identified species were *B. stearothermophilus* (9), *B. subtilis* (4), *B. brevis* (3), *B. pumilus* (3), *B. alcalophilus* (2), *B. badius* (2), *B. firmus* (2) and *B. lentus* (2). Four important proteolytic isolates of *Bacillus* were selected for molecular identification. The isolates were confirmed as *Bacillus subtilis* strain B20 (L/P/2/1), *B. subtilis* strain PB18 (D/P/3/1), *Bacillus* sp. strain BVC38 (D/P/3′/2) and *B. amyloliqefaciens* strain Egy25 (B/N/3′/1). Except B/N/3′/1 all the conventional identification was in accordance with the molecular identification as the isolate B/N/3′/1 was conventionally identified as *B. pumilus* (B/N/3′/1). All the isolates showed positive proteolytic activities on skim milk agar and the zone ratio was in between 2.61 ± 0.44 and 6.42 ± 0.95. These isolates could be commercially utilized in the tannery and detergent industries for their proteolytic activity.

Introduction

The tannery industry is one of the important industries in Bangladesh. About 150 tannery units i.e. 90% of the whole country are located at Hazaribagh of Dhaka. Bangladesh produces approximately 100 -150 million sq. feet of raw hides and skins, about 85% of which is exported in crust and finished form. The rest is used for producing leather goods to cater to the domestic market. The tanneries at Hazaribagh and its neighborhood reportedly discharge liquid and solid wastes. For the interaction between bacteria and leather processing, information is required on the types of bacteria found in effluents of the leather processing industries (Aunstrup 1979). Bangladesh and other South Asian countries produce and export leather as one of the major items which are mostly being processed by chemical treatment, resulting in inferior quality of products as well as environmental pollution. Use of alkaline protease in leather processing would be eco-friendly. The enzymatic dehairing and bating of hides have been widely accepted as a safe and sound alternative to the chemical process (Manachini *et al.* 1988).

Microorganisms are being used as a tool for production of biochemical and biologically active compounds mainly because of their abundant growth, higher productivity and lower production cost. The application of bacterial proteases is more significant when compared to the proteases from other sources like fungal (Anwar and Saleemudin 2000). Bacterial alkaline proteases are characterized by their high activity at alkaline pH and their broad substrate specificity. Effluent

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from leather processing industries might give us a good source of proteolytic bacilli. The genus *Bacillus* has a number of industrial importance and a good source of protease. Therefore, the present study was undertaken to isolate and identify *Bacillus* spp. associated with tannery industries and having proteolytic potential.

Materials and Methods

Hazaribagh tannery industries of Dhaka Metropolitan city, Bangladesh were selected for the present study. Samples were collected from the four selected stages *viz.* soaking, liming, deliming and bating. Sample water was collected in sterile plastic bottles sterilized with alcohol. The pH of the collected samples was measured in the laboratory by a pH meter (HM-31P, DKK-TOA Corp., Japan). Nutrient agar (NA) and peptone yeast extract glucose agar (PYG) media were used for the enumeration and isolation of aerobic heterotrophic bacteria present in samples. The pH of the medium was adjusted to 8.5. Enumeration and isolation of aerobic heterotrophic bacteria were carried out by serial dilution technique (Greenberg *et al.* 1998). The inoculated plates were inverted and incubated at 37°C for 48 hrs in an incubator (Memmert GmbH + Co Kg 8540 Sehwabach). After 48 hrs of incubation the plates having well discrete colonies were selected for counting. Using colony counter (Digital colony counter, DC-8 OSK 100086, Kayagaki, Japan) the developed colonies were counted.

Preliminary selection of the isolates was made on the basis of their distinctive colony morphology. Heat-shock test was done for confirmation of spore former bacteria representing *Bacillus* spp. Bacteria grown on three different protein based media (Gelatin medium, Coagulated egg medium and Skim milk agar medium) were used for their proteolytic activity (Wandersman *et al.* 1986). For conventional identification important biochemical tests were carried out *viz.* carbohydrate fermentation, catalase, deep glucose agar, tyrosine degradation, egg-yolk lecithinase, starch hydrolysis, methyl red, nitrate reduction, citrate utilization, oxidase etc. (Collins and Lyne 1984, Eklund and Lankford 1967, Peltier *et al.* 1959 and Bryan 1950). Following Bergey's Manual (Sneath *et al.* 1986) conventional identification was done. Proteolytic activity was determined by the zone ratio on skim milk agar (SMA). For this purpose 1 ml of sterilized milk was mixed with nutrient agar in sterilized Petri plate and allowed to solidify. Each of the isolates was point inoculated on SMA plate using sterilized inoculation needle and incubated at 37°C for 24 hrs. The isolates forming clear zone around the colonies were determined by mm scale. The following formula was used to determine the zone ratio.

Zone ratio = Zone diameter (mm)/Colony diameter (mm)

Potential four proteolytic isolates were taken for molecular identification based on 16S rDNA sequence analysis. For the partial amplification of 16S rDNA gene the following primer pairs were used- 5'-16S rRNA: CCAGACTCCTACGGGAGGCAGC, 3'-16S rRNA: CTTGTGCGGGC CCCCGTCAATTC. Supernatant of heat lysed cell suspension was used as the source of template DNA for PCR amplification of 16S rRNA gene. The following temperature and conditions were maintained for PCR amplification.

	Initial denaturation at	95ºC	For	5 min
30 cycles	Denaturation at	94ºC	For	1 min
	Annealing at	55ºC	For	30 sec
	Extension at	72ºC	For	1 min
	Final extension at	72ºC	For	10 min

After completion of cycling program, the reactions were held at 4ºC. The amplified products were separated electrophoretically on 1% agarose gel. DNA bands were observed on UV-transilluminator and photographed by a Gel Documentation system (Microdoc DI-HD, MUV21-254/365, Cleaver Scientific). The sequence generated from automated sequencing of PCR products were analyzed through NCBI-BLAST database (http://blast.ncbi.nlm.nih.gov/) and rRNA BLAST (http://bioinformatics.psb.ugent.be/cgi-bin/rRNA/blastform.cgi) programs to find out possible similar organism through alignment of homologous sequences.

Results and Discussion

Colour, pH and temperature of the samples are shown in the Table 1. Temperature of the collected samples ranged from 31 to 32°C. The pH of the samples ranged in between 7.52 and 12.11. The maximum pH (12.11) was found in the liming stage while the minimum (7.52) was recorded in the soaking stage. Both NA and PYG were found to be suitable for enumeration and isolation of bacteria. The bacterial load ranged between 1.34×10^5 and 3.44×10^5 cfu/ml on NA and 1.04×10^5 and 6×10^5 cfu/ml on PYG, respectively (Table 2). The bacterial counts among the replicates were found to vary and the maximum mean bacterial count was observed in the bating stage ($3.68 \pm 1.90 \times10^5$cfu/ml) while the minimum was in the deliming stage ($1.33 \pm 0.27 \times10^5$ cfu/ml). Primarily, 70 bacterial isolates were isolated based on their different colonial morphology. Based on heat shock test (Thiel *et al.* 1999), 27 isolates were selected for identification and proteolytic potential. All the isolates showed positive proteolytic activity on three protein based media *viz.* coagulated egg albumin, gelatin (data not shown) and casein.

Table 1. Colour, pH and temperature of the collected samples.

Stage	Colour	pH	Temperature (°C)
Soaking	Brown	7.52	32
Liming	Deep green	12.11	31
Deliming	Greyish green	8.93	31
Bating	Grey	8.42	32

Table 2. Bacterial count of different leather processing stages.

Replicates	cfu/ml of the collected samples in different leather processing stages							
	Soaking		Liming		Deliming		Bating	
	NA	PYG	NA	PYG	NA	PYG	NA	PYG
01	1.34×10^5	2.22×10^5	2.39×10^5	2.13×10^5	1.75×10^5	1.04×10^5	1.53×10^5	1.76×10^5
02	1.39×10^5	2.76×10^5	2.23×10^5	1.89×10^5	1.37×10^5	1.68×10^5	3.90×10^5	6.00×10^5
03	1.45×10^5	2.39×10^5	2.11×10^5	1.72×10^5	2.01×10^5	1.37×10^5	3.44×10^5	4.39×10^5
04	1.72×10^5	2.56×10^5	2.57×10^5	1.83×10^5	1.48×10^5	1.21×10^5	2.15×10^5	2.55×10^5
Mean ± Sd	$1.48 \pm 0.17\times10^5$	$2.48 \pm 0.23\times10^5$	$2.33 \pm 0.19\times10^5$	$1.89 \pm 0.17\times10^5$	$1.65 \pm 0.29\times10^5$	$1.33 \pm 0.27\times10^5$	$2.76 \pm 1.10\times10^5$	$3.68 \pm 1.90\times10^5$

Table 3. Important biochemical tests for conventional identification of the bacteria associated with tannery industry effluents.

Sample source	Isolate No.	Biochemical profile								Provisionally identified bacteria
		Voges Proskauer	Methyl red	Starch	Citrate	Propionate	Phenylalanine	Tyrosine	Lecithinase	
Soaking	S/N/1/1	–	–	+	+	–	–	–	–	*Bacillus stearothermophilus*
	S/N/1/3	+	+	+	+	–	–	–	+	
	S/N/2/1	+	+	+	+	–	–	–	+	
	S/N/2′/2	+	+	+	+	–	–	–	–	
	S/P/1′/1	+	+	–	+	+	–	–	–	
	S/P/1′/3	+	+	–	-	–	+	–	–	*B. lentus*
	S/P/5′/1	–	–	–	+	–	–	–	–	
	S/P/1/1	+	+	–	-	–	–	–	–	*B. alcalophilus*
	S/N/2′/1	–	–	–	+	–	–	+	+	*B. brevis*
	S/P/5/2	+	–	+	+	–	–	–	–	*B. badius*
	S/P/3/1	–	+	–	+	–	–	–	–	*B. pumilus*
Liming	L/N/2/1	+	+	+	+	–	–	–	–	*B. stearothermophilus*
	L/N/2′/1	+	+	+	+	–	–	–	–	
	L/N/3/1	+	+	+	+	–	–	–	–	
	L/P/4/1	+	+	+	+	–	–	–	–	
	L/P/2/1	+	+	–	-	–	–	–	–	*B. subtilis*
Deliming	D/N/4′/1	–	+	–	+	–	–	+	+	*B. brevis*
	D/P/3′/3	–	+	–	+	+	–	+	+	
	D/N/3/2	–	+	–	+	+	–	+	+	*B. firmus*
	D/P/3′/1	–	+	–	+	–	–	+	+	
	D/N/3′/1	–	+	–	+	–	–	+	+	*B. pumilus*
	D/P/3′/2	+	+	–	+	–	–	+	+	*B. subtilis*
	D/P/3/1	+	+	+	+	–	–	–	–	
	D/P/1/1	–	+	–	+	–	–	+	+	
	D/P/3/2	–	+	–	+	–	–	+	+	*B. alcalophilus*
	D/P/1/2	+	+	–	+	–	–	+	–	*B. badius*
Bating	B/N/3′/1	+	+	–	+	–	–	+	+	*B. pumilus*

+ for positive, - for negative result.

All the isolates were found to be Gram positive and spore formers. Important biochemical tests for conventional identification are shown in Table 3. Considering all the available morphological, microscopic and biochemical characteristics and following Bergey's Manual (Sneath *et al.* 1986) the isolated strains were conventionally identified. Selected 27 isolates belonged to the genus *Bacillus.* Under *Bacillus* there were 7 distinct species *viz. B. stearothermophilus*, *B. subtilis*, *B. brevis*, *B. pumilus*, *B. alcalophilus*, *B. badius*, *B. firmus* and *B. lentus* (Table 3). Saha *et al.* (2011) reported different *Bacillus* strains and one *Micrococcus* strain in the tannery effluents. Species frequency of the isolated *Bacillus* spp. is shown in Fig. 1. *Bacillus stearothermophilus* was found to be dominant among the isolated species.

Table 4. Protease activities on skim milk agar (SMA) medium measured as zone ratio.

Serial No.	Bacteria	Colony diameter (mm) (Mean ± Sd)	Zone diam. (mm) (Mean ± Sd)	Zone ratio (Mean ± Sd)
01	*Bacillus stearothermophilus*	11.00 ±2.65	33.00 ± 6.23	3.07 ± 0.60
02	*B. stearothermophilus*	11.67 ± 1.53	31.00 ± 1.00	2.68 ± 0.31
03	*B. stearothermophilus*	4.33 ± 0.58	19.00 ± 1.00	4.45 ± 0.75
04	*B. brevis*	7.33 ± 1.53	19.67 ± 1.53	2.73 ± 0.35
05	*B. stearothermophilus*	8.67 ± 0.58	31.00 ± 2.65	3.60 ± 0.57
06	*B. alcalophilus*	4.67 ± 1.16	29.67 ± 6.51	6.42 ± 0.95
07	*B. stearothermophilus*	4.00 ± 1.00	14.00 ± 2.65	3.54 ± 0.29
08	*B. lentus*	5.67 ± 1.53	31.33 ± 4.62	5.68 ± 0.82
09	*B. pumilus*	9.00 ± 1.00	29.79 ± 2.08	3.31 ± 0.18
10	*B. badius*	11.33 ± 1.53	33.33 ± 2.89	3.14 ± 0.26
11	*B. lentus*	4.67 ± 1.16	23.00 ± 2.65	5.03 ± 0.62
12	*B. stearothermophilus*	4.67 ± 0.58	18.67 ± 1.53	4.05 ± 0.68
13	*B. stearothermophilus*	4.67 ± 0.58	19.00 ± 2.65	4.10 ± 0.61
14	*B. stearothermophilus*	5.33 ± 1.16	18.67 ± 0.58	3.64 ± 0.97
15	*B. subtilis*	4.00 ± 1.00	16.33 ± 2.89	4.14 ± 0.48
16	*B. stearothermophilus*	4.67 ± 0.58	19.00 ± 1.73	4.13 ± 0.81
17	*B. firmus*	13.33 ± 1.53	35.33 ± 2.08	2.67 ± 0.24
18	*B. pumilus*	10.67 ± 2.08	32.67 ± 3.22	3.10 ± 0.37
19	*B. brevis*	10.00 ± 1.73	34.33 ± 3.79	3.47 ± 0.48
20	*B. subtilis*	9.67 ± 2.08	32.00 ± 5.57	3.33 ± 0.30
21	*B. badius*	16.33 ± 1.53	40.67 ± 4.51	2.66 ± 0.23
22	*B. subtilis*	10.33 ± 1.53	40.49 ± 2.65	3.92 ± 0.26
23	*B. alcalophilus*	10.33 ± 1.53	26.67 ± 3.22	2.61 ± 0.44
24	*B. firmus*	11.33 ± 1.53	37.00 ± 2.65	3.30 ± 0.47
25	*B. subtilis*	9.67 ± 1.16	33.67 ± 0.58	3.51 ± 0.37
26	*B. brevis*	10.33 ± 1.53	35.67 ± 1.53	3.49 ± 0.36
27	*B. pumilus*	8.66 ± 1.53	30.67 ± 8.08	3.50 ± 0.33

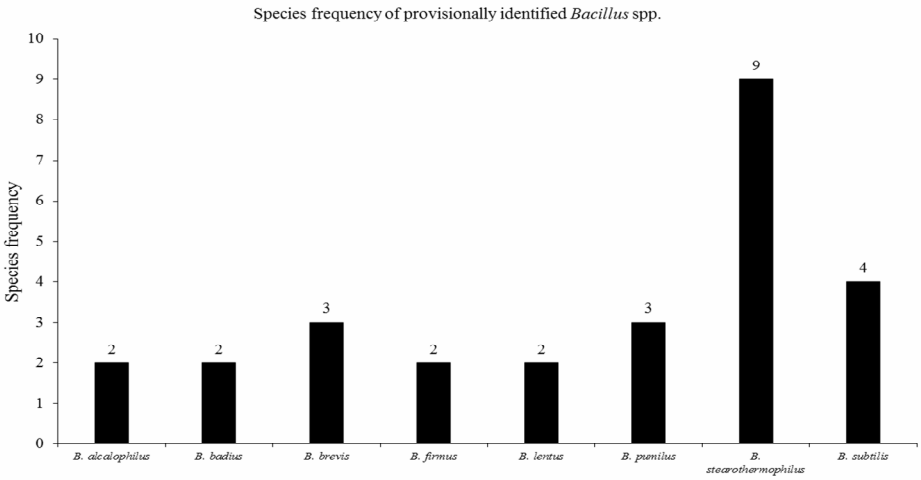

Fig. 1. Species frequency of provisionally identified *Bacillus* spp.

Protease activity on skim milk agar is shown in Table 4. All the isolates showed positive protease activities on casein with the zone ratio ranging between 2.61 ± 0.44 and 6.42 ± 0.95. Zone ratio of more than 5 was observed in *B. lentus* (S/P/5′/1 and S/P/1′/3) and *B. alcalophilus* (S/P/1/1). The highest zone ratio (6.42 ± 0.95) was observed in case of *B. alcalophilus* (S/P/1/1) which was isolated from the soaking stage. Saha *et al*. (2011) reported proteolytic bacteria in the tannery effluents with the zone ratio ranged in between 1.5 and 5.8. In the present study, the isolated *Bacillus* strains were found to be better zone ratio (2.67 to 6.42) than the report of Saha *et al*. (2011). Skim milk agar plate method was demonstrated to be effective in the screening of large number of proteolytic microorganisms (Wandersman *et al*. 1986, Lin *et al*. 1992).

Table 5. Comparison between conventional and molecular identification of selected isolates.

Isolate No.	Conventional identification	Molecular identification			
		Scientific name	Strain	Identity match (%)	Max. coverage score
L/P/2/1	*Bacillus subtilis*	*B. subtilis*	B20	89	309
D/P/3/1	*B. subtilis*	*B. subtilis*	PB18	97	861
D/P/3′/2	*B. subtilis*	*Bacillus* sp.	BVC38	91	579
B/N/3′/1	*B. pumilus*	*B. amyloliqefaciens*	Egy25	98	994

There are numerous reports about different strains of *Bacillus* including *B. alcalophilus, B. licheniformis, B. subtilis* and *B. thermobacter* to produce alkaline protease (Kelly and Fogarty 1976, Shah *et al.* 1986, Manachini *et al.* 1988, Takii *et al.* 1990). Hameed *et al.* (1996) reported *Bacillus subtilis* isolated from tannery waste which could produce alkaline protease. Padmapriya *et al.* (2012) isolated *Bacillus* sp. from green mussel collected from Kanyakumari coast, having high protease activity.

Usually *B. subtilis* is considered to be non-pathogenic to human. Therefore, three better proteolytic strains (L/P/2/1, D/P/3/1 and D/P/3′/2) were selected for molecular identification.

Similarly, one *B. pumilus* (B/N/3′/1) was selected for molecular identification. From the Fig. 2 we could see that, Lane M is the 1.0 kb ladder and lanes 1-4 are representing 4 different bacterial isolates *viz.* L/P/2/1, D/P/3/1, D/P/3′/2 and B/N/3′/1. In the gel approximate size of the amplified DNA band was 600 bp. The bacterial isolate L/P/2/1 was identified as *Bacillus subtilis* strain B20, the isolate D/P/3/1 was identified as *B. subtilis* strain PB18, the isolate D/P/3′/2 was identified as *Bacillus* sp. strain and the bacterial isolate B/N/3´/1 was identified as *B. amyloliqefaciens* strain Egy25 (Table 5). Among four isolates three matched with their conventional identification except *B. pumilus* (B/N/3′/1). In case of molecular identification the conventionally identified *B. pumilus* was found to be *B. amyloliqefaciens* strain Egy25.

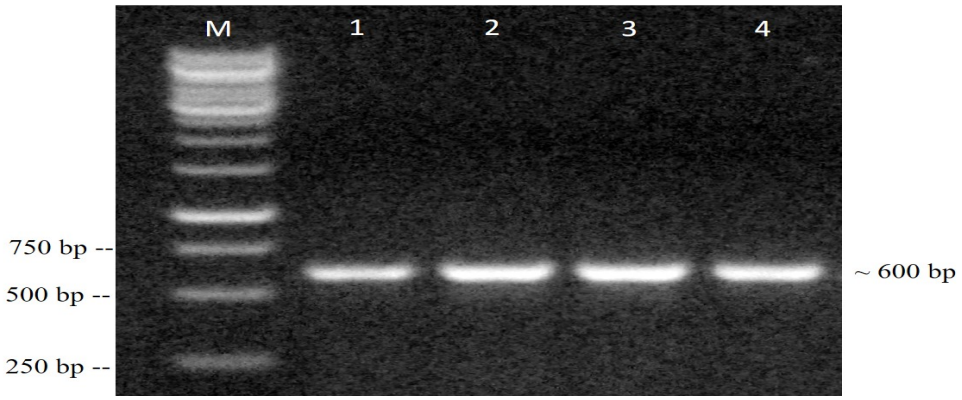

Fig. 2. PCR amplification of part of the 16S rRNA gene.

At present enzymes find huge application in various industries. In tannery, alkaline protease has been involved in soaking and bating of skin. Although proteolytic enzymes are produced by many microorganisms, only a few bacteria and fungi could secrete high quantity of proteases capable of extensive *in vitro* degradation of native milk protein casein. Extracellular proteases are known as the most secretary enzyme of the genus *Bacillus* (Nishiya and Imanaka 1990). Due to fast growth rate and easy of genetic manipulation, bacteria and their proteases have been receiving good attention.

The present findings showed that a varied species of *Bacillus* was found to be associated with the leather processing industries. The isolated *Bacillus* spp. clearly indicated a significant variety of proteolytic activity and these *Bacillus* spp. would be a good source of proteases for leather processing industries. More researches are to be needed for these isolated bacteria and optimization of their enzyme production as well as commercial utilization.

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