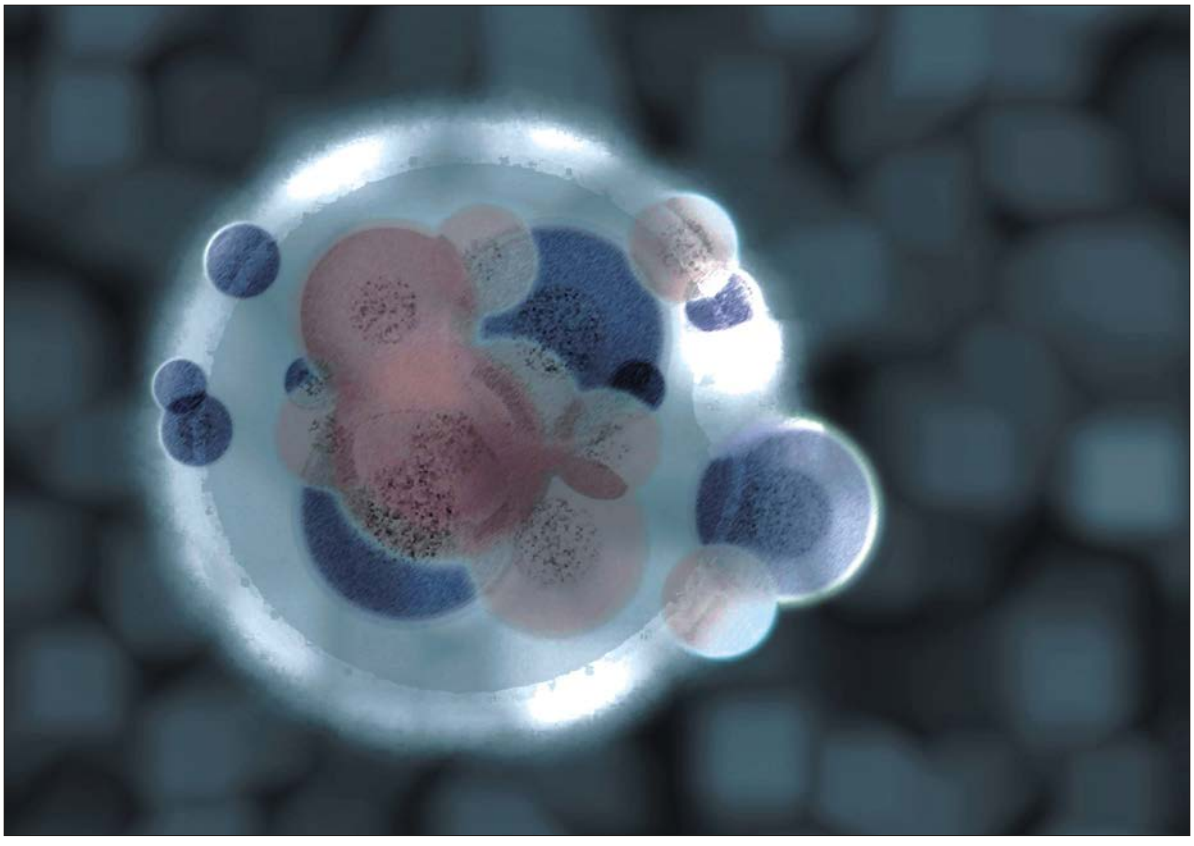

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Keratinolytic Activity of Some Newly Isolated *Bacillus* Species

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Abstract: Eight bacillus bacteria newly isolated from the effluents of tannery and poultry farms using a feather enrichment technique were identified on the basis of 16S ribosomal RNA gene sequence analysis, physiological and carbohydrates assimilation tests. Of them, 3 isolates were revealed as the strains of *Bacillus licheniformis*, two as *B. cereus* group and one each of *B. subtilis*, *B. borstelensis and B. sphericus*. Most of them demonstrated significant levels of keratinolytic protease on keratinous substrates (feather meal or hair keratin) in basal medium as a sole source of carbon, nitrogen and sulphur at 37°C and pH 8.0 in shake culture. Supplementation of yeast extract and molasses with feather in the basal medium increased the enzyme activity by most of the bacillus cultures. Commercial feather meal supported higher activity than that of commercial keratin powder. Of the bacillus species, *B. subtilis* MZK-7 and three strains of *B. licheniformis* displayed higher levels of keratinolytic activity under comparable conditions. The enzyme activity was found to be a function of cultivation times by different bacillus cultures. *B. borstelensis* MZK-6 had reached to its maximum activity after 32 h while the others did the same after 48 to 60 h on feather meal. The hydrolysis of synthetic peptides tested suggests that, among others, the enzymes from *B. licheniformis strains* and *B. subtilis* MZK-7 possess high levels of chymotripsin like (active towards Suc–Ala-Ala-Ala-pNA) and proteinase-K, elastase and subtilisin like (active towards Suc-Ala-Ala-Pro-Phe-pNA) protease activities. The protease inhibition studies with the enzymes from *B. licheniformis* strains and *B. subtilis* MZK-7 demonstrated the enzyme as serine protease while that of from *B. borstenlensis* MZK-6 and two strains *B. cereus* group demonstrated the neutral protease. The present results will be a useful basis for future studies on biotechnological production and application of keratinolytic enzymes.

Key words: Keratinolytic enzymes, 16S rDNA, *Bacillus licheniformis* MZK

INTRODUCTION

Keratins are insoluble proteins of rigid structure due to the presence of the high degree of cross-linking by disulfide bonds, hydrogen bonding and hydrophobic interactions[1] and hence extremely resistant to degradation by common proteolytic enzymes. Of proteinase enzymes, keratinase (E.C. no. 3.4.99.11) is important for hydrolyzing keratin containing substrates such as hair, feather and collagen[2]. The properties, secretion and use of microbial keratinases have recently been reviewed[3]. Keratinolytic proteases offer considerable opportunities for a low energy consuming technology for bioconversion of poultry feathers from a potent pollutant to a nutritionally upgraded protein feed staff for live stock. Of the 2 types of keratin, beta-keratin is rich in glycine, alamine, serine and specially lysine so after degradation by keratinase, hair, wool and feather can be used as a source of valuable amino acids and can consequently be used as animal feed preparation[4]. Keratinases have got a particular role in unhairing of hairs from the skins[5,6]. The alkaline protease preparation with keratinase has a potential application in leather manufacture. Since, keratinases could be of also interesting for the pharmaceuticals, cosmetics and leather industries, it is worth to search for potential microorganisms producing these enzymes.

Currently, a large proportion of the commercially available proteases is derived from bacillus strains although several fungal sources are being increasingly employed[7]. Bacillus have special advantages due to their ability to secrete large amounts of protein directly into the medium[8]. Among them, *B. licheniformis* strains appear to be potential candidates for industrial enzyme production. The secretion of alkaline serine protease is a frequent phenomenon amongst the different isolates of *B. licheniformis*[9,10]. The most effective keratin degrading strains in the bacillus genus belong to *B. licheniformis*. The keratinase of *B. lichneformis* PWD-1 is a serine protease that effectively degrades feather keratin. When

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PWD-1 is grown on feathers, subtilisin-like enzymes are also induced[11-17]. Although keratinases demonstrates a potential for its unique technical applications, its activities and yields have to be improved in order to make it feasible for industrial applications. In addition, the mechanism for enzymatic hydrolysis of insoluble keratin remains to be elucidated. Recently, the cooperative action of two enzymes (disulfide reductase and protease D-1) from a new isolate of *Stenotrophomonas* sp. involved in keratin biodegradation has been reported[18]. Hence, it is important to perform more studies with bacillus and their enzymes for the studies of keratin hydrolysis.

In this report, we detail the isolation and characterization of some bacillus species producing keratinolytic protease growing on feather meal or keratin as a sole source of carbon and nitrogen . The report includes the fermentation of keratinolytic enzymes and their characteristics profiles.

MATERIALS AND METHODS

Isolation of bacillus from enriched culture: For isolation of bacillus bacteria that produces keratinolytic protease, samples were collected from the feather-decomposed soil (effluents) from poultry farm and leather manufacturing industries (Tanneries), Dhaka, Bangladesh. About 100 g of these effluent wet soil samples were kept in a plastic container along with 2 g of chopped chicken feather (about 3 cm size) homogeneously mixed and incubated at room temperature. The samples were kept moistened with water at 2 days interval for 2 weeks.

From this sample, about 1 g partially degraded feather-soil mix was to inoculate in a 500 mL Erlenmeyer flask containing 100 mL of Peptone–yeast extract medium (per liter of solution: Peptone 10 g; yeast extract, 5.0 g; NaCl, 5.0 g; pH.7.0) for 24 h at 37°C in an orbital shaker at 100 rpm. Similar samples were also inoculated in peptone –KNO_3 mediuni (per liter of solution: bacto peptone, 5 g; meat extract, 3.0 g; KNO_3, 80 g; pH 7.7) in glass stopper bottle under partial anaerobic conditions for 48 h at the same temperatures specially to facilitate growth of *Bacillus licheniformis*[19].

Screening of proteolytic bacillus bacteria: Suitable dilution of this culture sample was spread on nutrient agar plates . The plates were incubated at 37°C and the number of colonies was counted after 24 and 48 h of incubation for all bacteria. These were then grouped on the basis of Gram staining reactions, catalase and oxidase reactions, typical spore formation and physiological tests related to genus bacillus[19]. Nineteen purified bacterial isolates were thus selected and were preserved at −70°C in 10% glycerol solution for long term storage. A milk-agar plate was then prepared for primary screening of bacteria that could produce proteases. Bacterial isolates were grown on Skimmed Milk Agar (SMA) (per liter of solution: skimmed milk, 100 g; agar, 20 g in 50 mM phosphate buffer, pH-7.0) at 37°C. Of them, eight bacteria which produced clear halos around its growth were thus selected for identification and characterization. *B. licheniformis* ATCC 9945a was used as a reference culture during this study.

The carbohydrates assimilation test of the isolates along with reference strain *B. licheniformis* ATCC 9945a were performed by API 50 CHB (bioMeriuex) carbohydrates testing instruction system

Identification by 16S rRNA gene sequence: Genomic DNA of the bacteria was isolated according to an established protocol[20]. Gene fragments specific for 16S rRNA–coding regions were amplified by PCR as described previously[21] using primers, 20F (5′-GAGTTTGATCCTGGCTCAG, positions 9-27) and 1500R (5′–GTTACCTTGTTACGACTT, positions 1509-1492). The numbering of positions in the rRNA gene fragments were based on the *E. coli* numbering system[22] (accession numberVOO348). Amplified 16S rRNA products were purified by standard protocol and sequenced with an ABI PRISM big dye Terminator cycle sequencing Ready Reaction Kit on an ABI PRISM model 310 genetic analyzer. The following 6 primers were used for complete sequencing: 20F, 1500R, 520F (5′–CAGCAGCCGCGGTAATAC; positions 519-536), 520R (5′-GTATTACCGCGGCTGCTG; positions 536-519), 920F (5′-AAACTCAAATGAATTGAGTTT: positions 907-926) and 920R (5′–CCGTCAATTCATTTGAGTTT; positions 926-907).

The sequence data was examined by BLAST Homology Search for identity test[23]. Multiple alignments of the sequences were carried out with the program CLUSTAL W (version 1.7)[24]. The distance matrices of the aligned sequences were calculated by using the two parameters method of Kimura[25]. The neighbour-joining method[26] was used for constructing a phylogenetic tree. The robustness for individual branches was estimated by bootstrapping with 1000 replicates[27].

Production of keratinolytic enzymes: Each of the bacillus isolate was cultivated in a basal medium (per litre of solution: NaCl, 0.5 g; $MgCl_2.6H_20$, 0.1 g; $CaCl_2$, 0.06 g; KH_2PO_4, 0.7 g; K_2HP0_4, 1.4 g) containing keratinous substrates: feather meal (Itochu Feed Meal, Japan), 10.0 g or keratin (α- keratin of hair) powder (Tokyo Kassei Co. Japan), 8.0 g; pH 7.5) as the only source of nitrogen, carbon and sulphur. The supplementation of nitrogen

(yeast extract), 0.1 g and carbon sources (molasses), 1.0 g together was also tested in presence or absence of keratinous substrates under identical conditions. Cultivation was done with 5 mL of 7-8 h grown inoculum (in nutrient broth) of the respective bacterial cultures in 100 mL above liquid medium in a 500 mL Erlenmeyer flask at 37°C under shaking (100 rpm) in a thermostated water bath for 60 h. The samples were withdrawn at 32, 48 or 60 h and centrifuged at 5000 rpm at 6°C for 20 min. The supernatants were preserved at 4°C and assayed for protein and enzymes.

Assay of keratinolytic protease: Azocasein hydrolysis was used as an alternative to the azo-keratin hydrolysis.

This assay was determined using azocasein (Sigma, USA) as substrate in a 2.0 mL microfuge tube with some modification the previous method[28]. The enzyme solution (400 μL) was incubated with 200 μL of 1.5% azocasein in 0.05 mM Tris-HCl buffer (pH–8.0) at 37°C for 30 min. The reaction was terminated by addition of 1.4 mL of TCA (Trichloro acetic acid) (10%). After 15 min, the reaction mixture was centrifuged at 10,000 g for 10 min and absorbance was read at 440 nm against a control in a spectrophotometer (U-2001, Hitachi, Japan). The control was treated in the same way, except that TCA was added before incubation. One unit of caseinolytic activity was defined as an increase of 0.01 absorbance units per min under the given conditions.

The activity against keratin powder (hair keratin) was performed as Gradisar *et al.*[29] with slight modification. One milliliter of 0.05 M Tris-HCl (pH 7.5) buffer containing 20 mg of keratin suspension was incubated with 250 μL of the enzyme solution. The reaction was terminated by addition of 500 μL of TCA. All other treatment conditions were same as above except that absorbance was read at 280 nm. The result was taken as an average of two replicas.

The protease activity against synthetic substrates was assayed according to the method of Kobayashi *et al.*[30]. The synthetic substrates were Suc-Ala-Ala-Pro-Phe-pNA and Suc-Ala-Ala-Ala-pNA (Sigma, Wisconsin, USA) The assay mixture contained 50 mM Tris-HCl (pH 8), 1 mM $CaCl_2$, pH–8.0 and 2 mM synthetic substrate. The reaction mixture was incubated at 30°C for 30 min. The reaction was terminated by adding of 5% citric acid and the activity was measured at 420 nm. One unit of enzyme activity was defined as the amount of the enzyme that liberated one micromole of p-nitro aniline per min under the conditions of the assay. The enzyme unit was thus calculated based on a standard graph prepared (y: 2.0195x – 0.1663; R^2: 0.9927) with p-nitro aniline. The protein concentration was assayed by following Bradford method[31] using comassie blue reagent.

Determination of type of protease by inhibitor: The effects of protease inhibitors on the proteolyic activity of the feather degrading bacillus were measured by azocasein hydrolysis. Culture supernatants taken at peak activity were used. Phenymethanesulphonylfluoride (PMSF, 2 mM) (Wako Pure Chemicals Ltd., Japan), EDTA (5 mM) (Wako Pure Chemicals Ltd., Japan) and 1, 10-phenanthroline (10 mM.) (Sigma, USA) were used to inhibit serine and metallo-proteinase. The control treatment consisted of azocasein without inhibitors. The percentage of each type of protease was calculated as follows: % of specific protease-$(A–A_i/A)$ 100 where, A is the total activity (without inhibitor), A_i is caseinolytic activity determined when a specific inhibitor was present. The effect of 2 inhibitors (PMSF and 1, 10-phenanthroline) together on the enzymes was also tested. The result was taken as an average of two replicas.

RESULTS

Isolation and identification of *Bacillus* species producing proteolytic enzymes: The feathers which were incubated with hair decomposed-soil-effluents in the laboratory for 2 weeks were found to be partially degraded. The samples were subsequently cultured in the yeast–peptone liquid culture medium. Suitably diluted culture was transferred onto nutrient agar for isolation of single colonies which were grouped on the basis of phenotypic (including gram staining reactions, catalase and oxidase reactions, typical spore formation) and physiological characteristics tests related to genus bacillus (including citrate, nitrate, propionate, anaerobic growth and growth at 50 and 60°C) (results are not shown). Nineteen pure representative bacillus-like cultures thus were isolated and selected.

These pure cultures were then tested for their proteolytic capability on SMA medium containing casein as the major protein source. Of the 19 isolates tested, eight demonstrated clear halos (resulted from the hydrolysis of casein) around the colonies on milk agar suggesting proteolytic activity (results are not shown). These cultures were then subjected to identification by carbohydrates assimilation and 16S rRNA gene sequence analysis and API 50 CHB tests.

Carbohydrates assimilation pattern of *Bacillus* species: The strains of *B. licheniformis* (*Bl*) showed a high correlation with *B. licheniformis* ATCC 9945a on the utilization of carbohydrates in API 50 CHB test protocol (Table 1). Out of 49 carbohydrates, *Bl* ATCC grew on

Table 1: Some characteristics of growth and carbohydrates assimilation in API CHB 50 test of different *Bacillus species*

Bacillus strains	Growth rate in nutrient broth		API 50 CHB test results			
	6 h	8 h	Particular sugar assimilated by *B. licheniformis*	Particular sugar not assimilated by *B. licheniformis*	Total score of positive reactions[1]	No. of assimilating carbohy-drates (out of 49)
B. cereus Grp MZK-1	+ + +	+ + + + (Fast)			10	15
B. licheniformis MZK-3	+	+ + (Slow)		D-xylose, Rhamnose	23	27
B. licheniformis MZK-4	+ + +	+ + + + (Fast)	Xylitol	Inositol	23	29
B. licheniformis MZK-5	+ + +	+ + + + (Fast)	Melibiose	N-acetyl glucosamine	25	29
Brevibacillus borsteleusis MZK-6	+	++ (Slow)			18	8
B. subtilis MZK-7	+ + +	+ + + + (Fast)			26	28
B.licheniformis-ATCC 9945a	+	+ + (Slow)			23	30
B. cereus Grp MZK-9	+ + +	+ + + + (Fast)			15	19

Observation were recorded for 3 times in nutrient broth at 37°C during 6 to 8 h level

[1]Based on semi-quantitative way of API 50 CHB instruction. The results of carbohydrates assimilation test were recorded after 24 h of incubation

30 while *Bl* MZK-3, *Bl* MZK-4 and *Bl* MZK-5 did so on 27, 29 and 29, respectively. These *B. licheniformis* strains also demonstrated the utilization of propionate which is known to be one of the specific test for *B. licheniformis*[19]. Although they are from the same species, distinct variation was observed with assimilation of the sugars (xylitol, d- xylose, rahamonose, melibiose, inositol and N-acetyl glucosamine) as shown in the Table 1. *Bl* MZK-3 could not assimilate d-xylose and rahamnose as contrast to other 3 strains. Similarly, *Bl* MZK-4 could not utilize inositol but it grew on xylitol as contrast to other 3 *Bl* strains. Finally, *Bl* MZK-5 could not utilize N-acetyl glucosamine but it utilized melibiose as contrast to other 3 strains of *B. licheniformis*. Two isolates of *B. cereus* MZK-1 and *B. cereus* MZK-9 assimilated a total of 15 and 19 sugars, respectively.

Variation was also observed with the growth of *B. licheniformis* strains in nutrient broth at 37°C. A qualitative indication of the repeated observations during inoculum preparation for 8 h culture with shaking at 100 rpm was observed at least for three times and recorded. The growth rate of *Bl* MZK-4 and *Bl* MZK-5 were faster found on the basis specific growth rate (result is not shown) than *Bl* MZK-3 and *Bl* ATCC cultures.

Finally, examination of 16S r RNA gene sequence analysis of the strains revealed 3 isolates as the strains of *B. licheniformis*(MZK-3, MZK-4 and MZK-5*)*, 2 isolates as *B. cereus* group (MZK-1 and MZK-9), one each of *B. subtilis* MZK-7, *B. borstenlenisis* MZK-6 and *B. sphericus* MZK-6 (Fig. 1).

Production of keratinolytic protease: Each of the eight bacillus species were grown in basal medium with or without feather meal or keratin powder as a sole source of carbon, nitrogen and sulphur at 37°C, pH 7.5. The bacillus species showed good growth (as observed) and protein synthesis (2.22 to 4.77 g L^{-1}) with a varying level of keratinolytic activity (Table 2) on feather meal except

Table 2: Protease production is a function of cultivation time by different *Bacillus* species on feather meal

Bacillus strains	Enzyme activity (U mL^{-1})		
	36 h	48 h	60 h
B. cereus Grp MZK-1	5.2	4.3	0.0
B. sphaericus MZK-2	3.2	0.0	0.0
B. licheniformis MZK-3	35.0	71.3	78.5
B. licheniformis MZK-4	9.6	26.5	48.3
B. licheniformis MZK-5	25.6	59.7	50.6
B. borsteleusis MZK-6	50.1	2.3	1.8
B. subtilis MZK-7	92.1	97.1	90.5
B. licheniformis-ATCC 9945a	72.5	76.5	73.2
B. cereus Grp. MZK-9	25.2	11.6	7.3

B. sphericus MZK-2. The high activity was demonstrated by *B. subtilis* MZK-7 (64.6 U mL^{-1}) and *B. licheniformis* strains (31.9 to 57.1 U mL^{-1}) after 48 hrs of cultivation on feather meal. Maximum activity, however, was observed with most of the bacillus after 60 hrs of cultivation except *B. borstenleis* which showed its highest activity (50.1 U mL^{-1}) after only 32 hrs of cultivation (Table 1). Both strains of *B. cereus* group showed lower keratinolytic activity on feather meal (Table 3). Keratin powder as an inducer displayed relatively lower keratinolytic activity in the cultures as compare to feather meal with all bacillus species. On keratin, *B. subtilis* MZK 7 and *B. licheniformis* MZK-3 showed also higher activity as compare to other bacillus species. Supplementation of molasses and yeast extract with feather meal in the culture promoted higher keratinolytic activity by all bacillus species. An increase of as high as about 43, 34 and 20% was recorded with *B. licheniformis* ATCC 9945a, *B. subtilis* MZK-7 and *B. licheniformis* MZK-3, respectively after 48 h of cultivation.

Substrate specificity-hydrolysis of synthetic peptides, keratin and azocasein: The enzyme preparations from different bacillus species cultivated on feather meal was tested for their hydrolysis capability of different substrates (Table 4). Both casein and keratin were hydrolyzed by the bacillus bacteria except that of by *Brevebacillus* MZK-6. The latter did not hydrolyze

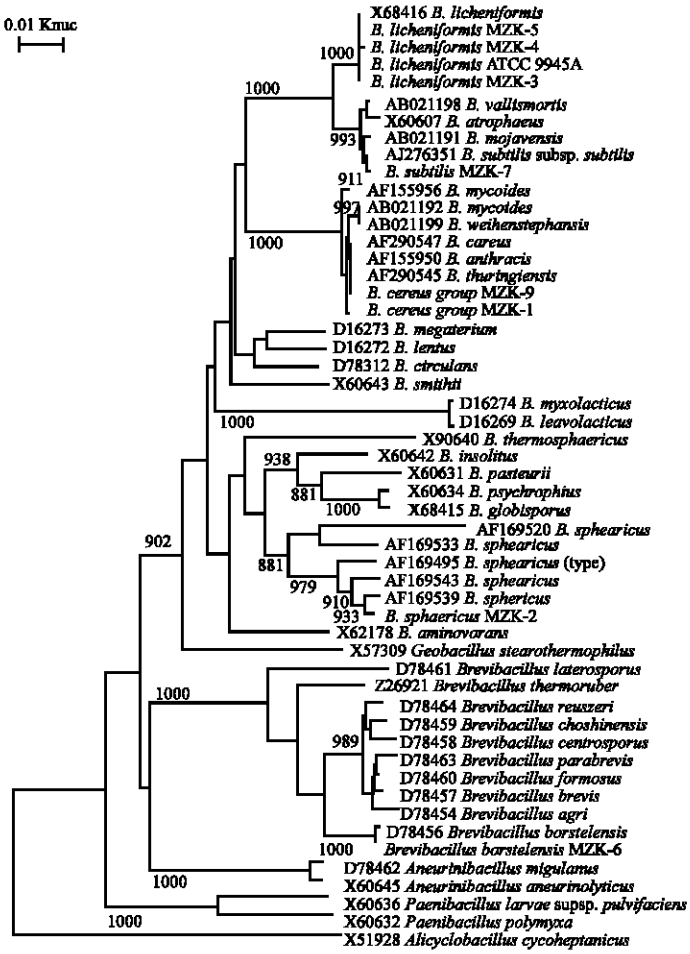

Fig. 1: Phylogenetic analysis based on the 16S rDNA sequence of *Bacillus* strains (*B. licheniformis* MZK-5, *B. licheniformis* MZK-4, *B. licheniformis* MZK-3, *B. subtilis* MZK-7, *B. cereus* MZK-9, *B. cereus* MZK-1, *B. sphaericus* MZK-2, *B. borstelensis* MZK-6) and a type strain (*B. licheniformis* ATCC9945a)

Table 3: Effect of inducers in basal medium on the synthesis of keratinolytic Protease by different *Bacillus* species

Bacillus culture	Feather (U mL^{-1})	Yeast+molasses +feather meal (U mL^{-1})	Keratin (U mL^{-1})
B. cereus Grp MZK-1	4.0	4.3	0.0
B. sphericus MZK-2	0.0	2.1	0.0
B. licheniformis –MZK-3	57.1	71.3	18.4
B. licheniformis-MZK-4	31.9	36.5	2.1
B. licheniformis-MZK-5	51.7	59.7	4.4
B. borsteleusis-MZK-6	2.1	2.3	0.0
B. subtilis MZK-7	64.6	97.1	35.3
B. licheniformis-ATCC 9945a	43.7	76.5	9.4
B. cereus Grp MZK-9	6.9	11.6	10.6

The results are mean values of 2 batches of cultivations after 48 h.

keratin. Each of the enzymes showed their hydrolysis activity with synthetic peptide Suc-Ala-Ala-Pro-Phe-p-Nitroanilide at varying level based on its enzyme concentration in the culture. Similarly, N-Suc-Ala-Ala-Ala-p-Nitroanilide was hydrolyzed by all enzymes except from the strains of *B. cereus* groups and *Brevibacillus* MZK-9.

Table 4: Hydrolysis of different substrates of protein/peptides by keratinolytic proteases from different *Bacillus* species grown on feather

Bacillus strains	Azocasein (U mL^{-1})	Keratin (U mL^{-1})	N-suc-Ala-Ala-Ala-pNA (U mL^{-1})	N-Suc-Ala-Ala-Pro-Phe-pNA (U mL^{-1})
B. cereus Grp- MZK-1	4.3	12.1	0.0	1.2
B. licheniformis MZK -3	71.3	33.9	30.2	131.3
B. icheniformis- MZK-4	26.5	10.0	9.3	116.8
B. licheniformis MZK -5	59.7	24.9	19.0	129.0
B. borsteleusis MZK -6	50.1	0.0	0.0	8.7
B. subtilis MZK -7	97.1	58.1	17.3	122.0
B. licheniformis ATCC 9945a	76.5	39.0	26.2	133.0
B. cereus Grp-MZK-9	11.6	23.2	0.0	60.4

In order to classify the types of proteinase produce, the inhibitory effects of PMSF, EDTA and phenanthroline on each enzyme is shown in the Table 5. The enzymes from *B. licheniformis* strains and *B. subtilis* were strongly

Table 5: Type of extracellular protease by different *Bacillus* strains on azocasein hydrolysis

Bacillus strains	Total activity (U mL^{-1})	Serine protease (%) (PMSF inhibition)	Neutral protease (EDTA inhibition)	Neutral protease (%) (Phenanthroline inhibition)
1B. licheniformis MZK-3	72.5	73.0	0.00	0.0
B. licheniformis-MZK-4	26.0	52.0	0.00	0.0
B.licheniformis-MZK-5	59.4	60.0	8.80	3.1
B. borstelensis-MZK-6	48.5	0.0	100.00	96.5
B. subtilis-HZK-7	106.6	84.0	12.46	4.2
B. licheniformis-ATCC 9945a	86.8	55.0	11.40	3.6
B. cereus Grp-MZK-9	11.5	0.0	76.80	85.9

inhibited by PMSF while a very negligible inhibition was observed with both EDTA and 1, 10-phenanthroline. On the contrary, the enzymes from *Brevibacillus* MZK-6 and strains of *B. cereus* group (MZK-1 and MZK-9) were strongly inhibited by both EDTA and phenanthroline but with no inhibition by PMSF. In presence of both inhibitors, no residual activity protease was observed.

DISCUSSION

The effluents of poultry farms as well as tanneries (Leather manufacturing industry) were rich in keratinous substrates such as poultry or animal (cow or goat) hairs. Further enrichment techniques in the laboratory finally aided to obtain keratin-degrading bacteria. These bacteria were tentatively identified as genus bacillus on the basis of phenotypic and physiological characteristics (including API 50 CHB).

The carbohydrates assimilation tests identified 4 groups of the bacillus based on closer scores of carbohydrates assimilation and oxidation reactions and total number of sugars utilized (Table 1). These are Group-1 (assimilation scores ranging between 23-25 for a total sugars of 27-30 by B-MZK-3, B-MZK-4, B-MZK-5, B-ATCC and B-MZK-7), Group-2 (score- 10-15 for a total sugars of 15-19 by B-MZK-1, B-MZK-9), Group-3 (Score 18 for a total sugars of 8 by B-MZK-6) and Group-4 (Score-3 for a single sugar by B-MZK-2) are suggested. Of all, propionate utilization test was demonstrated positive only with 4 cultures in group 1 isolating BMZK-7 apart from this group. Since propionate utilization is known to be specific for *B. licheniformis* species suggesting 3 cultures to be of the same species. It is of interest to note the differences amongst the *B. licheniformis* species exist in specific sugar (D-xylose, rhamnose, inositol, melibiose and xylitol and N-acetyl glucosamine) utilization and growth rates (Table 1). These informations may be useful for future studies with individual bacterium for their biotechnological studies and applications.

To confirm the identity at species level, these 8 bacteria were subjected to 16S rRNA gene sequence analysis. This studies led us to obtain 3 isolates (MZK-3, MZK-4 and MZK-5) demonstrating 99% identities of 1475 long bp with *B. licheniformis*, 2 isolates as *B. cereus* group (MZK-1 and MZK-9), one each of *B. subtilis* MZK-7, *B. borstenlenisis* MZK-6 and *B. sphericus* MZK-6.

The cultures thus isolated suggested strongly of bacteria that produces keratinolytic proteases in the cell free culture supernatants. Demonstration of keratinase enzymes on keratinous substrate as the sole source of carbon and nitrogen by *B. licheniformis* was also evidenced by other work[12,13,17]. In the present study, the strains of *B. licheniformis* and *B. subtilis* MZK-7 seem to be promising for keratinase production as compare to other bacillus species. However, the maximum activity as obtained here is a function of cultivation time by the bacteria tested. Even the 3 strains of *B. licheniformis* MZK produced maximum amount of enzyme under varied cultivation times. *Bl*-MZK-3 and MZK-4 still did not reach to its maximum after 60 h unlike other 2 strains of the same species. *Brevibacillus*–MZK-6 reached to its maximum level of keratinase synthesis after only 32 h and after which its activity was reduced drastically. Hence, further studies on the optimization of factors affecting enzyme production by individual bacterium will reveal their real potential as the enzyme producer. The hair keratin used in this study showed less potential as compare to feather meal as an inducer for keratinoytic protease. *B. sphericus* MZK-2 and *B. cerus* group MZK-1 although showed proteolytic activity on SMA medium but negligible or no kertinolytic activity was detected in the cell free extract. This may be due to its composition as alpha keratin is predominant in human hair where as feather meal contains mostly β- keratin. Similar results was obtained in the work of Yamamura *et al.*[18]. A significant level of enhancement of the enzyme activity level by different bacteria upon supplementation of the mineral medium and feather meal with molasses and yeast extract suggesting the option for development of appropriate medium composition for enzyme production.

Type of protease and substrate specificity: For all five major keratinolytic bacteria in the present study, serine protease activity accounted for approximately 65-90% of their total proteolytic activity with very lower level of neutral protease. In contrast, the 2 isolates (MZK-6 and

MZK-9) that did show poor keratinolytic activity possessed high levels of neutral protease activity; the two inhibitors together showed no residual activity suggesting the presence of two types of neutral or serine proteases producing bacillus.

Most of the high yielding producers of keratinase in the present study demonstrated its high activity towards synthetic peptide substrates such as Suc–Ala-Ala-Ala-pNA suggesting its properties as chymotrypsin like proteases. The activity towards Suc- Ala- Ala-Pro-Phe-pNA suggests the properties similar to proteinase-K, elastase and subtilisin. The present results will be a useful basis for future studies on biotechnological production and application of keratinolytic enzymes.

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