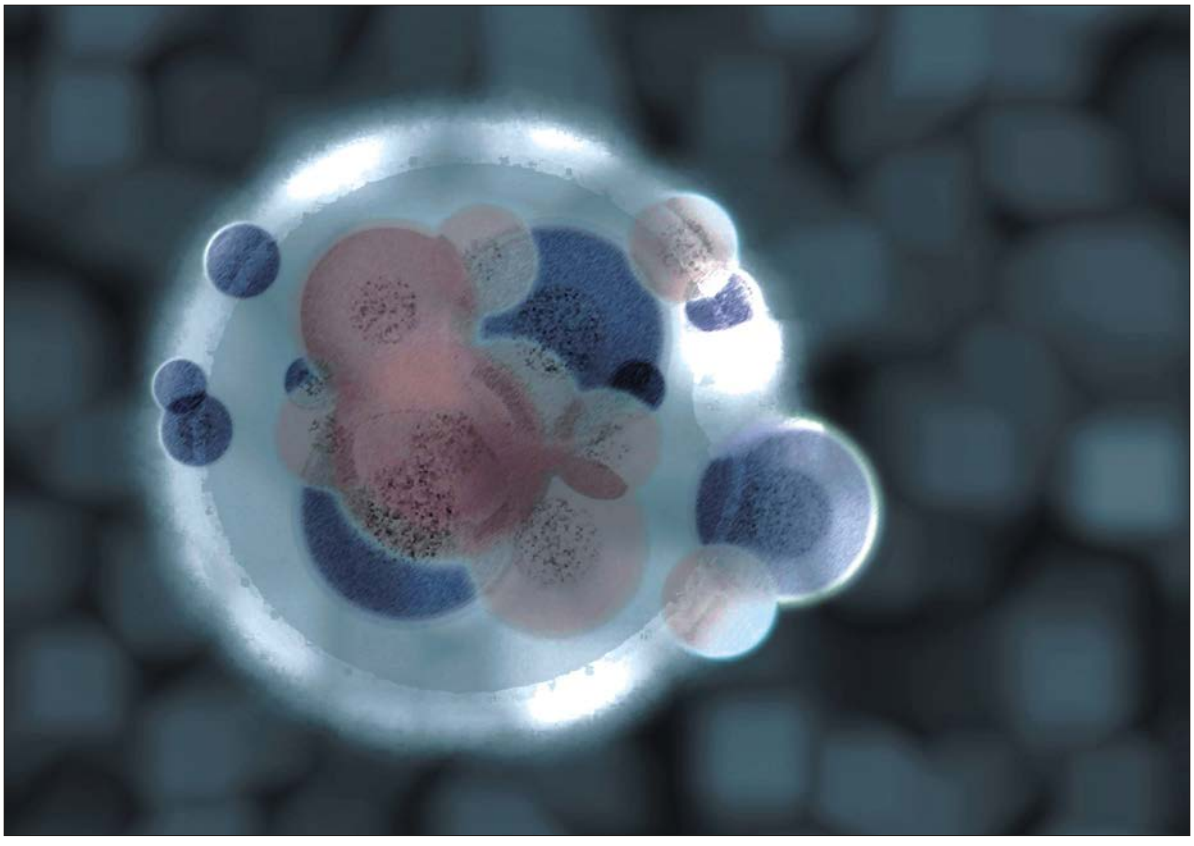

Journal of Biological Sciences

ISSN 1727-3048

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Journal of Biological Sciences 7 (4): 599-606, 2007
ISSN 1727-3048

Production and Partial Characterization of Feather-degrading Keratinolytic Serine Protease from *Bacillus licheniformis* MZK-3

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Abstract: A novel *Bacillus licheniformis* MZK-3 isolated from poultry wastes produced growth associated extracellular keratinolytic enzyme in the feather powder broth medium. The optimum temperature and initial pH for growth and enzyme production were 40°C and 8.0. The keratinolytic activity in enzyme preparations increased about 30 and 12%, when 1% (w/v) molasses and 0.1% (w/v) NH_4Cl was supplemented, respectively with the feather powder broth medium. The final 11-fold purified enzyme preparation showing the specific activity of 438.5 U mg•[1] was active and stable from pH 7.0 to 10.0 having the maximum activity at pH 9.0, thermostable at 30 to 50°C with 40°C as the optima. The half life of the enzyme at 50°C was 2 h and the activity was rapidly lost at 60°C or above. Experiment with protease inhibitors demonstrated that the enzyme was serine type as it was almost completely inhibited by PMSF. Both the crude and diluted purified enzyme preparations solubilized about 85% barbs of poultry feathers and 7% (w/w) of their native keratin after 12 h of incubation at 40°C, indicating that in practical application, this enzyme preparation is useful for promoting the hydrolysis of feather keratin and might have biotechnological potential involving keratin hydrolysis in the processing of poultry waste and leather industry.

Key words: *Bacillus licheniformis* MZK-3, keratin, keratinase, keratinolytic, feather-degradation, serine protease

INTRODUCTION

Feathers are produced as waste by-product at global poultry processing plants, reaching millions of tons per year with potential environmental impact (De Azeredo *et al*., 2006). Feathers are mainly constituted with • -kerotin which is a fibrous and insoluble structural protein extensively cross-linked by disulfide bonds, hydrogen bonding and hydrophobic interactions and thus becomes mechanically stable and extremely resistant to biodegradation by common proteases (Kojima *et al*., 2006; Ramnani *et al*., 2005; Thys *et al*., 2004). Feathers represent a potential alternative to expensive animal feedstuffs but the limitation arises from their poor degradation (Shih, 1993). The conventional process is expensive for thermo-energetic cost and furthermore, it destroys certain amino acids, yielding low nutritional quality feed with poor digestibility (Wang and Parsons, 1997). Keratinases are proteolytic enzymes in nature and are very important for hydrolyzing keratin containing substrates such as feathers, hair and collagen (Godfrey and West, 1996). Keratin of feathers and hair being degraded by the keratinase can be used as potential source of amino acids like glycine, alanine, serine, cysteine, proline and lysine for animal feed preparation and for many other applications and thereby, keratinase may offer a low energy consuming technology for bioconversion of poultry feathers from a potent pollutant to nutritionally upgraded protein feedstuff for livestock (De Toni *et al*., 2002; Hoq *et al*., 2005; Thys *et al*., 2004). Keratinase has also potential application in leather manufacturing due to its particular role in unhairing from the skin (Letourneau *et al*., 1998; Mukohopadhyay and Chandra, 1993). In addition, keratinolytic enzymes may have important use in biotechnological valorization of keratin-containing wastes generated from poultry and leather industries and may have prospective role in the development of nonpolluting process (Mukohopadhyay and Chandra, 1993; Onifade *et al*., 1998; Shih, 1993). Since keratinase could also be

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interesting for the pharmaceutical and cosmetic industries (De Azeredo *et al*., 2006; Gradisar *et al*., 2000), it is worth to study the production of efficient keratinase that imposes biotechnological significance in manufacturing industries and waste management.

Keratinolytic enzymes are produced by some species of *Bacillus* (Hoq *et al*., 2005; Kim *et al*., 2001; Kojima *et al*., 2006; Ramnani *et al*., 2005; Suntornsuk and Suntornsuk, 2003; Werlang and Brandelli, 2005), *Stretomyces* (Bressollier *et al*., 1999; Chitte *et al*., 1999; De Azeredo *et al*., 2006), *Xanthomonas* (De Toni *et al*., 2002), *Chryseobacterium* (Riffel *et al*., 2003), *Mycobacterium* (Thys *et al*., 2004), *Vibrio* (Sangali and Brandelli, 2000) etc. Recently, we isolated *B. licheniformis* MZK-3 with significant keratinolytic activity (Hoq *et al*., 2005). Optimization of parameters such as pH, temperature and media composition is important in developing the cultivation process for production of an enzyme. Despite many works that have been done to screen and characterize keratinolytic microorganisms, limited information is available about direct application of keratinolytic proteases or keratinases from bacteria. This paper shows the practical application of keratinolytic serine protease preparation from *B. licheniformis* MZK-3 for native feather degradation in addition with the optimization of production conditions and determination of some enzymatic properties. To the best of our concern, it is the first report showing the direct application of keratinolytic enzyme preparation from bacteria to native poultry feathers for degradation of keratin.

MATERIALS AND METHODS

Bacterial strain: The bacterium, *B. licheniformis* MZK-3, was isolated and identified by 16S rRNA gene sequence analysis in the previous study (Hoq *et al*., 2005). Stock cultures of the strain in nutrient broth (Difco Laboratories, Detroit, MI, USA) were stored at -70°C with 20% (v/v) glycerol in the Department of Microbiology, University of Dhaka, Bangladesh.

Inoculum preparation and enzyme production: A loopful of stock pure culture was transferred to a sterile screw capped test tube containing 5 mL of nutrient broth and incubated at 37°C in an orbital shaker for 24 h to obtain the fresh seed culture. This culture (at A_{660}; 1.0) was added to 100 mL feather powder broth (FPB) medium containing (g L^{-1}, pH 7.5) NaCl, 0.5; K_2HPO_4, 0.3; KH_2PO_4, 0.4; $MgCl_2.6H_2O$, 0.1; $CaCl_2.2H_2O$, 0.1 and feather powder, 10. The feather powder was used as the only source of carbon, nitrogen and sulphur. After incubation for 48 h at 37°C under shaking conditions (120 rpm), the growth was measured by cfu and the culture was harvested by centrifugation at 5000 rpm for 20 min at 4°C. The cell-free supernatant was used as crude preparation of enzyme for keratinolytic activity. Protein concentrations were determined according to the method of Bradford using protein assay reagent (Bio-Rad) with bovine serum albumin as the standard protein (Bradford, 1976).

Assay for keratinolytic activity: The keratinolytic activity of the enzyme preparations was determined using keratin (keratin powder) as substrate with slight modifications of the method previously used (Gradisar *et al*., 2000). In brief, 1 mL of 50 mM Tris-HCl, pH 7.5 containing 20 mg of keratin suspension was incubated with 250 μL of the enzyme solution for 30 min at 37°C with constant agitation at 120 rpm in a water bath. The reaction was stopped by adding 500 μL of 10% trichloroacetic acid (TCA) and then incubation at 4°C. After 30 min, the reaction mixture was centrifuged at 10,000 x g for 10 min and the supernatant was used to read the absorbance at 280 nm against a control. The control was treated in the same way, except that the TCA was added before the addition of enzyme solution. One unit of keratinolytic activity was defined as an increase of corrected A_{280} for 0.01 under the assay conditions. The result was taken as an average of three replicates.

Assay for proteolytic activity: The proteolytic activity of the enzyme was determined using azocasein (Sigma, USA) as substrate by the method described previously (Hoq *et al*., 2005). In brief, 400 μL of enzyme solution was incubated with 200 μL of 1.5% azocasein in 50 mM Tris-HCl, pH 7.5 at 37°C for 30 min. The reaction was stopped by addition of 1.4 mL of 10% TCA. After 15 min, the reaction mixture was centrifuged at 10,000 x g for 10 min. The proteolytic activity in the supernatant was measured at 440 nm against a control. In the control, TCA was added prior to addition of enzyme solution in the reaction mixture. One unit of proteolytic activity was defined as an increase of 0.01 absorbance under the described assay conditions.

Growth kinetics and enzyme production: The fresh seed culture (5% of total growth medium) of *B. licheniformis* MZK-3 was inoculated in the FPB medium (initial pH 7.5) and incubated at 37°C for 60 h under shaking at 120 rpm. The culture samples were withdrawn aseptically every 3 h and the growth along with keratinolytic activity was monitored as described above.

Optimization of cultural conditions for growth and enzyme production: To detect the influence of medium pH on growth and production of keratinolytic enzyme,

B. licheniformis MZK-3 was grown in the FPB at different initial pHs (5.0 to 11.0). The fermentation was carried out at 37°C for 42 h under shaking at 120 rpm. To investigate the effect of temperature on growth and keratinolytic enzyme production, fermentation was performed with the FPB medium (initial pH 8.0) at different temperatures (30, 35, 37, 40, 45, 50 and 55°C) for 42 h at 120 rpm. The growth and enzyme activity was quantified as described above.

Effect of different carbon and nitrogen sources on growth and enzyme production: The growth and production of keratinolytic enzyme were investigated by adding 1% (w/v) various carbon sources (glucose, fructose, sucrose, lactose and molasses) to the FPB medium, initial pH 8.0. To investigate the effect of various nitrogen sources on growth and the enzyme production, 1% (w/v) peptone, tryptone, combination of peptone and tryptone (0.5% of each) and 0.1% (w/v) NH_4Cl were supplemented to the FPB medium, initial pH 8.0. The growth and enzyme activity were monitored after fermentation at 40°C for 36 h under shaking at 120 rpm.

Partial purification of enzyme: The crude enzyme preparation (500 mL) was precipitated with $(NH_4)_2SO_4$ fractionations (0-20%, 20-40%, 40-60% and 60-80%) using standard method. The resulting precipitates were collected by centrifugation at 10,000 x g for 15 min at 4°C and dissolved in minimal volume of 20 mM Tris-HCl, pH 7.5 and dialyzed over night against the same buffer at 4°C. The fraction containing the keratinolytic activity was applied onto a Sephadex G-75 column (1.6×70 cm) previously equilibrated with 20 mM Tris-HCl, pH 7.5. The protein was eluted with 20 mM Tris-HCl, pH 7.5 at a rate of 1 mL min^{-1}. Fractions of 2 mL were collected to analyze the keratinolytic activity as described above. The active fractions were pooled to use for characterization by proteolytic activity.

Effects of pH and temperature on proteolytic activity and stability: To investigate the optimum assay pH of purified keratinolytic enzyme preparation, reactions for proteolytic activity were carried out in the range from pH 6.0 to 13.0 using the following different 50 mM buffers: KH_2PO_4-K_2HPO_4 (pH 6.0 to 7.5), Tris-HCl (pH 8.0 to 9.0), Na_2HPO_4-NaOH (pH 11.0 to 12.0) and Glycine-NaOH (pH 9.0 to 13.0). To determine the optimum assay temperature, the proteolytic reactions were performed at different temperatures ranging from 30 to 60°C at optimum pH. The pH stability of the enzyme was determined with a slight modification of the method previously described (Azad *et al.*, 2002). In brief, the purified enzyme preparation (100 μL) was incubated with 900 μL of 50 mM appropriate buffers (mentioned above) from pH 6.0 to 13.0 at 10°C for 24 h and the residual proteolytic activity was measured in the standard assay conditions. To determine the thermo stability, the purified enzyme preparation was pre-incubated at temperatures ranging from 30 to 70°C with 50 mM Tris-HCl, pH 8.0. The residual proteolytic activity was measured in the standard assay conditions at different time intervals.

Effect of protease inhibitors: For determination of protease type, the following inhibitors were added to the purified enzyme preparation: 2 mM PMSF (Wako Pure chemicals Ltd., Japan), 5 mM EDTA (Wako Pure chemicals Ltd., Japan) and 10 mM 1, 10 phenanthroline (Sigma, USA). After 30 min of pre-incubation at room temperature with each inhibitor in 50 mM Tris-HCl buffer (pH 8.0), the residual proteolytic activity was measured in standard assay conditions. The PMSF was dissolved in acetonitrile for stock.

Feather-degradation by crude and purified enzyme preparations: A 0.1 g of autoclaved native whole feather was incubated in the test tube containing l0 mL of either crude or purified enzyme preparations. After incubation for 12 h at 40°C, the test tubes were inspected for visible degradation of feathers against feather containing distilled water as a blank and the loss of dry weight of the feather was determined according to the method described by Bockle *et al.* (1995).

RESULTS AND DISCUSSION

Growth associated keratinolytic enzyme production: The growth kinetics of *B. licheniformis* MZK-3 along with enzyme production at 37°C and pH 7.5 using the FPB medium revealed the maximum level of keratinolytic activity (51 U mL^{-1}) at 42 h of cultivation (Fig. 1). Figure 1 shows that the organism entered the exponential phase after 6 h and the stationary phase started after 42 h. The enzyme production was coherent with the growth pattern and it increased with the increase of growth and thereafter remained almost static at optimal level along the stationary phase up to 57 h. The synthesis of this extracellular enzyme by *B. licheniformis* MZK-3 was inducible (Hoq *et al.*, 2005) and 1% feather powder in the FPB was sufficient for maximum growth and enzyme production (data not shown).

Effects of medium pH and cultural temperature on growth and production of keratinolytic enzyme: The growth and keratinolytic enzyme synthesis with *B. licheniformis*

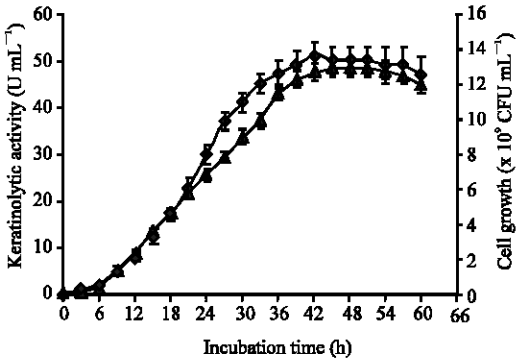

Fig. 1: Time course of growth and keratinolytic enzyme production by *B. licheniformis* MZK-3. The growth (▲) and keratinolytic activity (◆) were measured every 3 h during the cultivation. Data are the means ± SE (SE, standard error) of five independent experiments with comparable results. Bars = SE

MZK-3 was greatly influenced by the initial medium pH showing maximum production at slightly alkaline condition (pH 8.0) (Fig. 2a). Although similar trends were evident in *B. licheniformis* PWD-1 with optimum pH 9.0 for growth and keratinase production (Cheng *et al*., 1995), Wang and Shih (1999) reported neutral initial pH for the best production of keratinase with the same species. Temperature is an important environmental factor affecting the growth and production of metabolites by microorganisms (Banerjee and Bhattacharya, 1992). The optimum temperature for the growth and production of keratinolytic protease by *B. licheniformis* MZK-3 was 40°C (Fig. 2b). This temperature optima for maximum keratinolytic enzyme production by this organism is almost similar with that of by other *Bacillus* species (Cheng *et al*., 1995; Wang and Shih, 1999), but dissimilar with keratinolyitc *Vibrio* sp. kr2 (Sangali and Brandelli, 2000), *Lysobacter* sp. (Allpress *et al*., 2002) and *Streptomyces* sp. (De Azeredo *et al*., 2006), which showed optimum temperature for growth and keratinase production ranging from 20 to 30°C. The optimum growth temperature for *B. licheniformis* PWD-1 was previously reported as 50°C (Cheng *et al*., 1995; Wang and Shih, 1999). This temperature is significantly higher than that of *B. licheniformis* MZK-3, indicating that conditions for the growth and keratinolytic enzyme production may differ depending on the different strains of the same species.

Influence of carbon and nitrogen sources on growth and keratinolytic enzyme production: To optimize the culture media for keratinolytic enzyme production by *B. licheniformis* MZK-3, several carbon and nitrogen

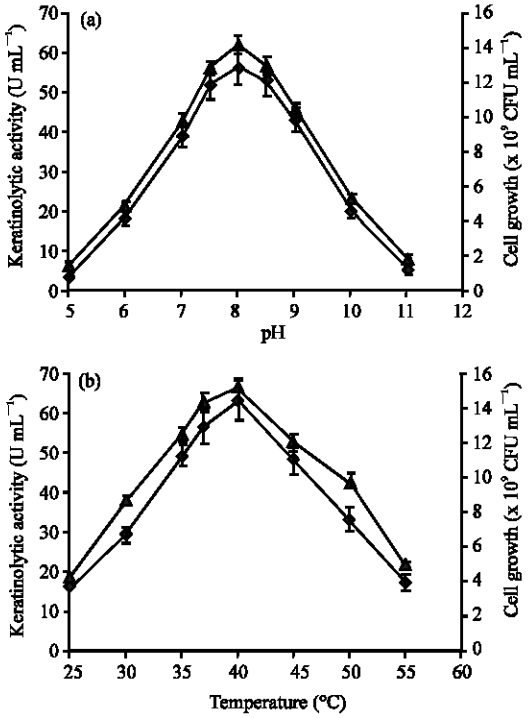

Fig. 2: Determination of optimum initial medium pH (a) and cultural temperature (b) for production of keratinolytic enzyme by *B. licheniformis* MZK-3. The growth (▲) and keratinolytic activity (◆) are shown in both panels. Data are the means ± SE of five separate experiments

Table 1: Effects of different carbon and nitrogen sources on growth and keratinolytic enzyme production[a]

Carbon/Nitrogen sources	Keratinolytic activity (U mL^{-1})	Growth (x 10^9 CFU mL^{-1})
Control[b]	60	14.3
Glucose	37	9.8
Fructose	41	10.5
Sucrose	39	9.9
Molasses	78	17.1
Peptone	59	14.2
Tryptone	60	14.3
Peptone + Tryptone	61	14.2
NH_4Cl	67	14.9

[a]Carbon and nitrogen sources were supplemented to the FPB medium as described in materials and methods. Data are typical of triplicate experiments with comparable results. [b]Fermentation was carried out in the FPB medium

sources were supplemented with the FPB medium. Table 1 shows that addition of molasses increased the keratinolytic activity in the enzyme preparation about 30% possibly due to its vitamin and mineral sources. Studies with different concentrations of molasses in the FPB medium showed that 1% molasses was sufficient to maximize the enhancement of the enzyme production (data not shown). However, further investigation is required to define the exact nature of molasses to cause

this enrichment. Although inhibition in keratinase production by molasses was observed through the study of Wang and Shih (1999), Cheng *et al.* (1995), however, reported significant increase in keratinase synthesis by *B. licheniformis* PWD-1. Table 1 further shows that glucose, fructose and sucrose inhibited growth and production of keratinolytic enzyme. This might be due to the repression of expression of gene for keratinase (Wang and Shih, 1999). Such type of inhibition by carbohydrate in the synthesis of keratinase was previously reported with *B. licheniformis* PWD-1 (Cheng *et al.*, 1995; Wang and Shih, 1999). In case of nitrogen sources, NH_4Cl influenced the enzyme production by increasing about 12% of keratinolytic activity (Table 1). Peptone, tryptone or their combination was not helpful to increase the keratinolytic activity in the FPB medium by this strain (Table 1) even though tryptone induced significant amount of keratinase activity in other *B. licheniformis* (Cheng *et al.*, 1995). It indicates that *B. licheniformis* MZK-3 in the FPB fermentation media preferentially used keratin rather than peptone or tryptone to produce keratinolytic protease. The combination of NH_4Cl (0.1%) and molasses (1%) supplemented to the FPB showed somewhat positive cumulative influence (keratinolytic activity ~82 U mL^{-1}) on enzyme production by this organism. These two nutrients showed no significant keratinolytic enzyme production when the feather powder was replaced by 1% polypeptone plus 0.5% yeast extract (data not shown). This result further supported the notion that the keratinolytic enzyme synthesized by *B. licheniformis* MZK-3 might be inducible only in the presence of keratinous substrate in the growth medium.

Although the production of proteases in complex growth media often promotes exuberant growth and high enzyme yields (Joo *et al.*, 2002), their expensive cost makes them unsuitable for a large-scale production. Some less expensive substrates, such as soybean meal, have been successfully used for alkaline protease or keratinolytic protease production (Cheng *et al.*, 1995; Joo *et al.*, 2002). The strain in this study produced higher yields in the FPB medium in which the feather powder had been used as good substrate for production of keratinolytic enzyme. The feather powder is natural keratinous substrate rather than synthetic ones commercially available, such as keratin azure or azokeratin. Moreover, in the presence of feather powder, molasses that is cheap and available as a by-product from different sugarcane industries has been shown to significantly increase the productivity of keratinolytic enzyme by *B. licheniformis* MZK-3.

Characterization of keratinolytic protease of *B. licheniformis* MKZ-3: The 60-80% $(NH_4)_2SO_4$ saturated fraction showing the keratinolytic activity was further purified by Sephadex G-75 column. The results of purification profile with keratinolytic activity of the enzyme preparation from *B. licheniformis* MKZ-3 are summarized in Table 2. The final 11-fold purified enzyme showed the specific activity of 438.5 U mg^{-1} with a typical yield of 21.2%.

To characterize the purified enzyme, the proteolytic activity was determined using azocasein as substrate because the proteolytic activity against azocasein well correlated with keratinolytic activity (data not shown). The enzyme was active and stable within a range of pH 7.0 to 10.0 having the maximum activity at pH 9.0 (Fig. 3). Figure 3 shows that the enzyme had the residual proteolytic activity about 85% at pH 7.0 to 10.0 and more than 50% at pH 11.0 after 24 h of treatment. The stability at alkaline conditions may fit this enzyme preparation as a potential enzyme in unhairing of skin in leather industries in addition with feather degradation since the unhairing step is usually performed at alkaline pHs (Azad *et al.*, 2002). The proteolytic activity was observed in the temperature range from 30 to 50°C with the optima at 40°C (Fig. 4a). The activity was thermo-stable at 40°C with the residual proteolytic activity more than 80% after 4h (Fig. 4b). The thermo stability for long time at the optimum temperature might be very important for the application of this enzyme. Figure 4b showed that the half life of the enzyme at 50°C was 2 h and at 60°C or above, the enzyme lost its activity rapidly. The half lives of this enzyme at various temperatures (at 60°C~13 min and at 70°C~7 min) resemble to keratinase from *Chrysosporium keratinophilum* but differ from keratinase of thermophilic *B. pseudofirmus* and *Chryseobacterium* sp. (Dozie *et al.*, 1994; Kojima *et al.*, 2006; Riffel *et al.*, 2003). The proteolytic activity was almost completely (90%) inhibited by PMSF, an inhibitor of serine protease and partially (15%) by EDTA, a metalloprotease inhibitor (Fig. 5). A neutral protease inhibitor (1, 10 phenanthroline)

Table 2: Purification of keratinolytic enzyme from *B. licheniformis* MZK-3[a]

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1} protein)	Purity (fold)	Yield (%)
Crude supernatant	685.0	27300.0	39.9	1.0	100.0
$(NH_4)_2SO_4$ fractionation (60-80%)	104.7	13349.0	127.5	3.2	48.9
Sephadex G-75	13.2	5787.6	438.5	11.0	21.2

[a]Keratinolytic activity was assayed in every step and data are typical of three independent experiments with comparable results

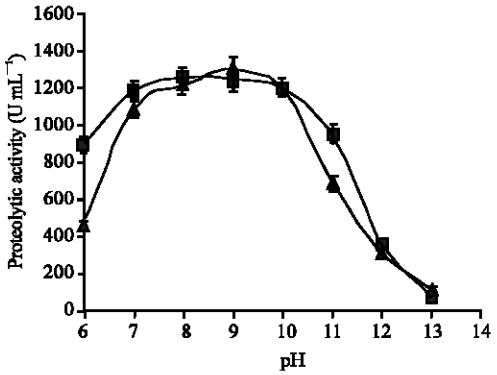

Fig. 3: Effect of pH on the activity (▲) and stability (■) of keratinolytic protease from *B. licheniformis* MZK-3. Data are the means ± SE of five independent experiments

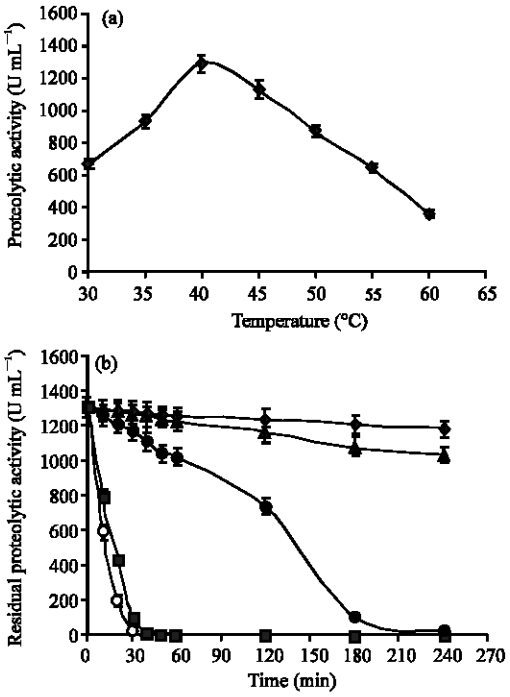

Fig. 4: Effect of temperature on the activity (a) and stability (b) of keratinolytic protease from *B. licheniformis* MZK-3. b, enzyme was incubated for inactivation at 30°C (◆), 40°C (▲), 50°C (●), 60°C (■), or 70°C (•) and residual proteolytic activity was measured. Data are the means ± SE of five independent experiments

had no effect on proteolytic activity. These results suggested that the keratinolytic enzyme of *B. licheniformis* MZK-3 was serine protease in nature. This property is common to most of keratinases from *B. licheniformis* PWD-1, *B. pseudofirmus* FA30-01 and

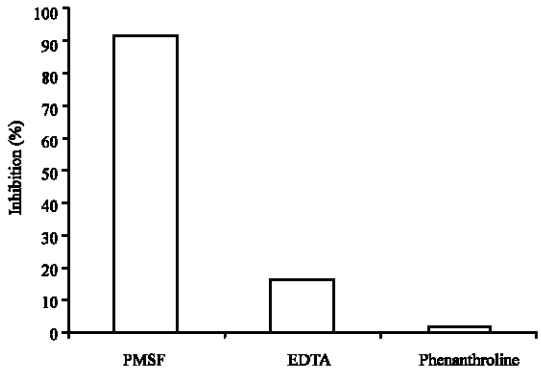

Fig. 5: Effects of protease inhibitors on the proteolytic activity of purified keratinolytic enzyme from *B. licheniformis* MZK-3. To calculate the inhibition rate compared to the control (without inhibitor), the residual proteolytic activity was measured. Data are typical of triplicate experiments with comparable results

B. subtilis (Kojima *et al*., 2006; Lin *et al*., 1995; Suh and Lee, 2001). However, keratinase may also be metalloprotease described from *Mycobacterium*, *Chryseobacterium* and *Lysobacter* (Allpress *et al*., 2002; Riffel *et al*., 2003; Thys *et al*., 2004) and the enzyme in this study may be influenced by some metal ions since EDTA had partial inhibition.

Native feather degradation by keratinolytic protease of *B. licheniformis* MZK-3: Both the crude and purified enzyme preparations were used to determine the ability to solubilize the intact native poultry feathers. Visible observation revealed that the crude enzyme preparations (60 U mL•^{1}) from *B. licheniformis* MZK-3 dissolved about 85% barbs of the feather (Fig. 6, tube 1) and that (41 U mL•^{1}) of *B. licheniformis* MZK-5 (Hoq *et al*., 2005) dissolved near 70% barbs (tube 2) compared to the control, indicating that feather degradation was concomitant with keratinolytic activity of the enzyme preparations. Almost the same pattern of barbs dissolution was observed with the diluted purified enzyme (60 U mL•^{1}) preparation from *B. licheniformis* MZK-3 (data not shown). The dissolution of keratin of native feathers by the crude and purified enzyme preparations was estimated by measuring the residual dry weight of nondegraded feathers. The crude and diluted purified enzyme preparations dissolved 7 and 8% dry weight of feathers, respectively (Table 3). However, purified enzyme preparation with about 3-fold keratinolytic activity dissolved about 17% dry weight of native feathers (Table 3). This result indicated that the crude and partially purified enzyme preparations synthesized by

Fig. 6: Solubilization of native feathers by crude keratinolytic enzyme from *B. licheniformis* MZK-3. Autoclaved poultry feathers were incubated with 10 mL keratinolytic enzyme preparations from *B. licheniformis* MZK-3 (tube 1) and *B. licheniformis* MZK-5 (Hoq *et al*., 2005) (tube 2). Tube 3 was used as control only with distilled water. Typical result of three repeated experiments is shown

Table 3: Degradation of native poultry feathers by crude and purified keratinolytic enzyme preparations from *B. licheniformis* MZK-3[a]

Keratinolytic enzyme preparations	Keratinolytic activity (U mL•[1])	Residual insoluble keratin (%)
Crude	60	93
Purified[b]	60	92
Purified[c]	200	83

[a]Native poultry feathers were treated with enzyme preparations as described in materials and methods. Data are typical of at least three repeated experiments. [b]Purified enzyme preparation was diluted to keratinolytic activity of 60 U mL•[1]. [c]Keratinolytic activity was adjusted to 200 U mL•[1] by dilution

B. licheniformis MZK-3 were significantly keratinolytic and capable to hydrolyze natural keratinous substrate and resembled with the keratinolytic activity of keratinases of *Streptomyces albidoflavus* and *S. pactum* (Bockle *et al*., 1995; Bressollier *et al*., 1999), which solubilized native keratin (native feather, hair keratin, keratin azure etc) up to 10-15% based on the dry weight loss.

In this study, several parameters for synthesis of keratinolytic enzyme by the *B. licheniformis* MZK-3 were optimized and some of the enzymatic properties were determined. It is an essential step for production and application of keratinolytic enzyme in research of non-polluting processing of wastes in the poultry and leather industries, despite recycling of feathers for production of feedstuff, fertilizer, glues and rare amino acids. Enhanced synthesis of keratinolytic protease by this strain using cheaper molasses with feather powder would be remarkable. Solubilization of native poultry feathers by the crude and purified enzyme preparations showed the biotechnological potential involving keratin hydrolysis in the processing of poultry waste and animal skin in leather industries. This work, however, further elucidated that for the evaluation of biotechnological application of the keratinolytic protease from *B. licheniformis* MZK-3 requires more detailed understanding of the factors that enable this enzyme for complete degradation of native keratinous substrates. Therefore, additional research will be done for the molecular characterization of this interesting enzyme along with bioconversion perspectives.

REFERENCES

Allpress, J.D., G. Mountain and P.C. Gowland, 2002. Production, purification and characterization of an extracellular keratinase from *Lysobacter* NCIMB 9407. Lett. Applied Microbiol., 34: 337-342.

Azad, A.K., H. Shibata and M.M. Hoq, 2002. Hide processing with alkaline protease from *Bacillus* sp. MA6. Bangladesh J. Microbiol., 19: 67-71.

Banerjee, R. and B.C. Bhattacharya, 1992. Extracellular alkaline protease of newly isolated *Rhizopus oryzae*. Biotechnol. Lett., 14: 301-304.

Bockle, B., B. Galunsky and R. Muller, 1995. Characterization of a keratinolytic serine proteinase from *Streptomyces pactum* DSM 40530. Applied Environ. Microbiol., 61: 3705-3710.

Bradford, M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein using the principle for protein-dye binding. Anal. Biochem., 72: 280-285.

Bressollier, P., F. Letourneau, M. Urdaci and B. Verneuil, 1999. Purification and characterization of a keratinolytic serine proteinase from *Streptomyces albidoflavus*. Applied Environ. Microbiol., 65: 2570-2576.

Cheng, S.W., H.M. Hu and S.W. Shen, 1995. Production and characterization of keratinase of a feather degrading *Bacillus licheniformis* PWD-l. Biosci. Biotechnol. Biochem., 59: 2239-2243.

Chitte, R.R., V.K. Nalawade and S. Dey, 1999. Keratinolytic activity from the broth of a feather-degrading thermophilic *Streptomyces thermoviolaeus* strain SD8. Lett. Applied Microbiol., 28: 131-136.

De Azeredo, L.A.I., M.B. De Lima, R.R.R. Coelho and D.M.G. Freire, 2006. Thermophilic protease production by *Streptomyces* sp. 594 in submerged and solid-state fermentation using feather meal. J. Applied Microbiol., 100: 641-647.

De Toni, C.H., M.F. Richter, J.R. Chagas, J.A.P. Henriques and C. Termignoni, 2002. Purification and characterization of an alkaline serine endopeptidase from a feather-degrading Xanthomonas maltophilia strain. Can. J. Microbiol., 48: 342-348.

Dozie, I.N.S., C.N. Okeke and N.C. Unaeze, 1994. A thermostable, alkaline-active, keratinolytic proteinase from Crysosporium keratinophilum. World J. Microbiol. Biotechnol., 10: 563-567.

Godfrey, T. and S. West, 1996. Industrial Enzymology. 2nd Edn. New York, N.Y: Macmillan Publishers Inc., pp: 3.

Gradisar, H., S. Kern and I. Friedrich, 2000. Keratinase of *Doratomyces microsporous*. Applied Microbiol. Biotechnol., 53: 196-200.

Hoq, M.M., K.A.Z. Siddiquee, H. Kawasaki and T. Seki, 2005. Keratinolytic activity of some newly isolated *Bacillus* species. J. Biol. Sci., 5: 193-200.

Joo, H.S., C.G. Kumar, G.C. Park, K.T. Kim, S.R. Paik and C.S. Chang, 2002. Optimization of the production of an extracellular alkaline protease from *Bacillus horikoshi*. Process Biochem., 38: 155-159.

Kim, J.M., W.J. Lim and H.J. Suh, 2001. Feather-degrading *Bacillus* species from poultry waste. Process Biochem., 37: 287-291.

Kojima, M., M. Kanai, S. Kitazume, A. Inoue and K. Horikoshi, 2006. Isolation and characterization of a feather-degrading enzyme from *Bacillus pseudofirmus* FA30-01. Extremophiles, 10: 229-235.

Letourneau, F., V. Soussotte, P. Bressollier, P. Brandland and B. Verneuil, 1998. Keratinolytic activity of *Streptomyces* sp. $S.K._{1-02}$: A new isolated strain. Lett. Applied Microbiol., 26: 77-80.

Lin, X., D.W. Kelemen, E.S. Miller and J.C.H. Shih, 1995. Nucleotide sequence and expression of kerA, the gene encoding a keratinolytic protease of *Bacillus licheniformis* PWD-1. Applied Environ. Microbiol., 61: 1469-1474.

Mukohopadhyay, R.P. and A.L. Chandra, 1993. Protease of a keratinolytic *Streptomycetes* to unhair goat skin. Ind. J. Ex. Biol., 31: 557-558.

Onifade, A.A., N.A. Al-Shane, A.A. Al-Musallam and S. Al-Zarban, 1998. A review: potentials for biotechnological applications of keratin-degrading microorganisms and their enzymes for nutritional improvement of feathers and other keratins as livestock feed resources. Bioresource Technol., 66: 1-11.

Ramnani, P., R. Singh and R. Gupta, 2005. Keratinolytic potential *Bacillus licheniformis* RG1: structural and biochemical mechanism of feather degradation. Can. J. Microbiol., 51: 191-196.

Riffel, A., F. Licas, P. Heeb and A. Brandelli, 2003. Characterization of a new keratinolytic bacterium that completely degrades native feather keratin. Archives Microbiol., 179: 258-265.

Sangali, S. and A. Brandelli, 2000. Feather keratin hydrolysis by a *Vivrio* sp. Kr2 strain. J. Applied Microbiol., 89: 735-743.

Shih, J.C.H., 1993. Recent development in poultry waste digestion and feather utilization. A review. Poultry Sci., 72: 1617-1620.

Suh, H.J. and H.K. Lee, 2001. Charaterization of keratinolytic serine protease from *Bacillus subtilis* KS-1. J. Prot. Chem., 20: 165-169.

Suntornsuk, W. and L. Suntornsuk, 2003. Feather degradation by *Bacillus* sp. FK46 in submerged cultivation. Bioresour. Technol., 86: 239-243.

Thys, R.C.S., F.S. Lucas, A. Riffel, P. Heeb and A. Brandelli, 2004. Characterization of a protease of a feather-degrading *Microbacterium* species. Lett. Applied Microbiol., 39: 181-186.

Wang, X. and C.M. Parsons, 1997. Effect of processing systems on protein quality of feather meal and hog hair meals. Poultry Sci., 76: 491-496.

Wang, J.J. and J.C.H. Shih, 1999. Fermentation production of keratinase from *Bacillus licheniformis* PWD-1 and a recombinant *B. subtilis* FDB-29. J. Indust. Microbiol. Biotechnol., 22: 608-616.

Werlang, P.O. and A. Brandelli, 2005. Characterization of a novel feather-degrading *Bacillus* sp. strain. Applied Biochem. Biotechnol., 120: 71-80.