

## Preliminary Study on Keratinase from Two Indonesian Isolates

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**Abstract.** Keratinases (E.C.3.4.99.11) constitute a group of enzymes capable of disrupting the highly stable keratin structure consisting of disulphide, hydrogen, and hydrophobic bonds in the form of  $\alpha$ -helices and  $\beta$ -sheets *B. licheniformis* MB-2 and *Bacillus sp.* MTS are two feather-degrading bacteria isolated from Tompaso crater at North Sulawesi and sulfuric land around Tangkuban Perahu in West Java. They were both capable of breaking down whole chicken feathers. In addition both isolates were capable of degrading other proteinous substrates rich in beta structure such as cocoon, silk, human hair and fish scales. Result of fermentation experiment implied that addition of nitrogen sources (0.02% yeast extract and 0.02% tryptone) to the basal medium increased keratinase production. Our experiments showed that keratinase production of *Bacillus sp.* MTS was higher and faster than that from *B. licheniformis* MB-2. Maximum extracellular keratinase activity of the enzyme derived from *B. licheniformis* was obtained during stationary phase at 72 h, while *Bacillus sp.* MTS was reached at 48 h. Disulfide reductase activity also detected in the extracellular fluid of *Bacillus sp.* MTS. The maximum condition for extracellular keratinase activity was 55°C and the enzyme showed two maximum pHs : pH 8.0 and pH 10. The zymogram analysis indicated sixth protein bands of 17, 25, 32, 53, 96 and 122 kD which were able to hydrolyze gelatin substrate in-situ.

**Key Words :** *Bacillus*, feather, keratinase, disulfide reductase

### Introduction

Insoluble and hard-to-degrade animal proteins are ubiquitously present throughout animal bodies e.g. nails, horns, hair, wool and feather. Feather wastes are generated in large quantities as a byproduct of commercial poultry processing. Feathers represent 5-7% of the total weight mature chickens and its made up primarily of keratin. Keratin is resistant to common proteolytic enzymes and poorly digested by most organism. However, keratin does not accumulate in nature. Biological processes may be accomplish its to removal. The mechanical stability of keratin and its resistance to biochemical degradation depend on tight packing of the protein chains in  $\alpha$ -helix ( $\alpha$ -keratin) or  $\beta$ -sheet ( $\beta$ -keratin) structures and linkage of these structures by disulfide bonds. Digestive enzymes, such as trypsin and pepsin are not effective in keratin degradation. Only proteinase K is well known for its high keratin hydrolysing activity. Enzymes capable of degrading keratin is known as having keratinolytic activity.

A number of microorganisms have been reported could degrade different sources of

keratin, mainly bacteria, actinomycetes, saprophytic and dermatologist fungi have been reported to exhibit keratinolytic properties. Keratinases from many bacteria have been isolated and characterized. For instance, keratinase from *Bacillus sp.* (Zerdani et al., 2004; Werlang and Brandelli, 2005), *B. pumilus* (Huang et al., 2003); *B. licheniformis* (Ramnani et al., 2005; Lin et al., 2001), *Chryseobacterium sp.* (Riffel et al., 2003), *Pseudomonas* (Brandelli and Riffel, 2006), *Microbacterium* (Thys et al., 2004)), *Streptomyces* sp. (Bockle and Muller, 1997; Bressolier et al., 1999; Letourneau et al., 1998; Ya-peng et al., 2007) and *Stenotrophomonas* sp. (Yamamura et al., 2002).

The majority of reports on keratinases that have been characterized focused on their proteolytic action. Nonetheless, the reduction of disulfide bonds may have a significant role in the degradation of keratin. Thiol formation by *Vibrio* strain kr2 (Sangali and Brandelli, 2000) and *Chryseobacterium* sp. strain kr6 (Riffel et al., 2003) have suggested the presence of disulfide reductase in the culture grown on keratin feather. Extracellular keratinolytic activity of *B. licheniformis* RG1 was synergistically enhanced 4-

fold by addition of intracellular disulfide reductases (Ramnani et al., 2005). Reduction of disulfide bonds was also observed for *Streptomyces pactum* grown on feather (Bockle and Muller, 1997), *Streptomyces* sp. S.K<sub>1-02</sub> (Letourneau et al., 1998) and *S. fradiae* on wool (Shama and Berwick, 1991). Though some reports are available on the presence of disulfide bond-reducing factors in keratin utilizing microorganism, researcher rarely have focused on the isolation and characterization of disulfide bond-reducing enzymes with respect to keratin degradation.

In our previous study, we isolated two *Bacillus* sp. from hotspring water and sulphuric soil that are capable to degrade native-chicken feather, they are *B. licheniformis* MB-2 and *Bacillus* sp. MTS. In this study, we compared the ability of both *Bacillus* sp. to choose the best isolate and medium which potential as keratinase producer. Preliminary characterized the extracellular keratinase also presented.

## Materials and Methods

### Bacterial strains

Thermophilic bacteria *B. licheniformis* MB-2 was isolated from hotspring water at Tompaso Mountain-North Sulawesi (Toharisman et al., 2005), while mesophilic bacteria *Bacillus* sp. MTS was isolated from sulphuric soil at Tangkuban Perahu Mountain-West Java.

### Media and culture conditions

We prepared different basal medium because both isolate could not growth at the same medium. The basal medium used for maintenance and growth of *B. licheniformis* MB-2 was feather meal medium containing the following : 0.1% K<sub>2</sub>PO<sub>4</sub>, 0.1% NaCl, 0.01% MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.07% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.01% yeast extract, 0.01% bacto tryptone, 1% feather meal, 0.5% gelrite and 1.5% bacto agar. The pH was adjusted to 6.5 in a 0.5 L erlenmeyer flask at 55°C and agitated at 100 rpm in an incubator shaker.

The basal medium used for *Bacillus* sp. MTS was feather meal medium containing the following : 0.03% K<sub>2</sub>PO<sub>4</sub>, 0.05% NaCl, 0.01% MgCl<sub>2</sub>.6H<sub>2</sub>O, 0.05% NH<sub>4</sub>Cl, 1% feather meal

and 2% bacto agar. The pH was adjusted to 7.5 in a 0.5 L erlenmeyer flask at 37°C and agitated at 100 rpm in an incubator shaker. The cultures was filtered and then centrifuged at 4°C and 10.000 rpm for 10 min to harvest enzymes-containing supernatant.

To produce large amounts of keratinase, different carbon sources such as 0.02% (w/v) yeast extract and tryptone together and 0.02% (w/v) yeast extract added separately to the keratin medium. The initial cell number used was 5 x 10<sup>6</sup> cells/ml. These culture were incubated on a rotary shaker (100 rpm) at 37°C and 55°C. The enzyme production was monitored for 5 days. The result reported here were from triplicates experiments.

### Assay of keratinolytic activity

Keratinase activity was assayed with azokeratin as a substrate. Azokeratin was synthesized based on the methodology described for azoalbumin Riffel et al. (2003). The reaction mixture contained 100 µl enzyme, 400 µl azokeratin (10 g azokeratin/l) and 400 µl 50 mM tris buffer pH 8.0. The mixture was incubated for 15 minutes at 37°C; the reaction was stopped by addition of trichloroacetic acids to a final concentration of 100 g/l. After centrifugation at 10.000 rpm for 5 min, the absorbance of supernatant was determined at 440 nm. One unit of enzyme activity equaled to the amount of enzyme that caused 0.01 absorbance's changes at 440 nm for 15 min at 37°C.

### Assay of disulfide reductase activity

The disulfide reductase activity was measured as described by Serrano et al. (1984) with modifications. Reaction mixture in a final volume of 1.5 ml contained: 100 µmol of Tris buffer (pH 8.5), 2.5 µmol GSSG (oxidized glutathione), 0.5 µmol EDTA and an adequate quantity of enzyme. The mixture was incubated for 10 minutes at 37°C centrifuged at 1036xg for 10 min. A mixture of 50 µl supernatant was mixed with 100 µl 20 mM DTNB (5,5'-dithiobis-(2-nitrobenzoic acid) and 1350 µl Tris buffer (pH 8.5). Absorbance of the reaction product was determined at 405 nm. Enzyme control was prepared in similar manner without addition of substrate. One unit of sulphydryl reductase was equivalent to the amount of enzyme required to release 1 µmol of sulphydryl per ml per min at room temperature.

### Protein determination

Quantitative protein determination was measured spectrophotometrically at 595 nm with bovine serum albumin used as a standard protein (Waterborg, 2002).

### SDS-PAGE and Zymogram

To determine the molecular mass of enzyme, SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was carried out according to Laemmli's method (Walker 2002). After SDS-PAGE the gel was stained by silver staining according to Vorum method (Moertz et al., 2001). The acrylamide concentrations of the stacking and separating gels were 4% and 8% respectively. To prepare zymogram, gelatin (0.5%, w/v) in 50 mM Tris-HCl buffer (pH 8.5) was mixed into separating gels. After electrophoresis. The gel was washed with 2.5% (w/v) Triton X-100 for 60 min. After overnight of incubation in 50 mM Tris-HCl buffer (pH 8.5) at 55°C, the gel was stained with Coomassie brilliant blue R-250 for 30 min and then destained. Protease band appeared as clear zones on a blue background. Low molecular weight (LMW) used as a standard proteins. LMW contains phosphorylase b (97 kDa), bovine serum albumine (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and  $\alpha$ -Lactalbumin (14.4 kDa).

### Results and Discussion

Feather degradation by bacteria has been widely described for *Bacillus* spp., which appear to be ubiquitous bacteria in poultry waste and plumage of birds (Thys et al., 2004;

Burtt and Ichida, 1999). In our research, the complete degradation of whole feather by *B. licheniformis* MB-2 and *Bacillus* sp. MTS were observed in basal medium within two weeks at optimum conditions. The whole feather was fully degraded by *B. licheniformis* MB-2 at 12<sup>nd</sup> days incubation, while with *Bacillus* sp. MTS the native feather was completely degraded at fourth days of incubation (Fig.1). *B. licheniformis* PWD-1 was reported possesses ability to degrade native chicken feather completely at 37°C in 10 days, while with *Stenotrophomonas* sp. D-1 at 20°C this occurred within only 2.5 days. *B. licheniformis* RG1 was much more rapid, and complete degradation of keratin occurred within 24 h. Earlier studies with other microorganism have reported longer incubation periods of 48 h to 28 days (Ramnani and Gupta, 2004; Thys et al., 2004; Werlang and Brandelli, 2005; Allpress et al., 2002; Yamamura et al., 2002).

We also observed degradation of other keratinase substrates by both isolates. Measurement of keratinolytic activity was conducted to substrate weight loss. Table 1 showed the nature-keratin weight loss after two weeks incubation by *B. licheniformis* MB-2 and six days incubation by *Bacillus* sp. MTS.

The most easily accessible substrate was scales of fish which is, apparently, attacked also by non-specific proteases. With the hard keratin, the rate of hydrolysis corresponds roughly to the "hardness" i.e. cystine content. Feather, cocoon and silk were therefore cleaved more easily than human hair. Various cystine content were found on nature keratin, 15-22% in human hair, 12% in wool, 7% in feather, 5.2% in human nail, 3.2% in horse nail and 6.8% in horn (Wilson and Lewis, 2006; Goddard and Michaelis, 2008).

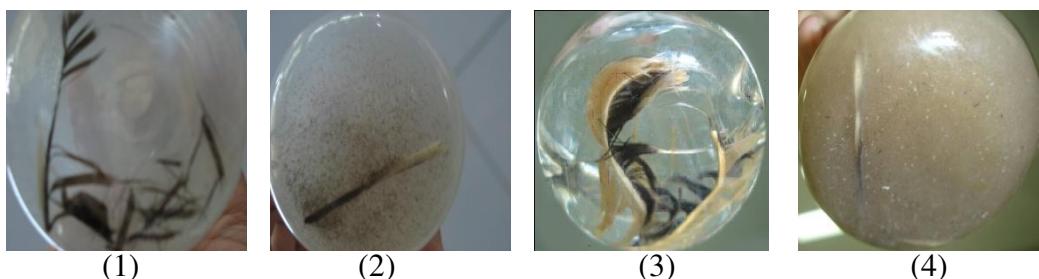


Figure 1. Complete degradation of whole feather by *B. licheniformis* MB-2 (1 & 2) and *Bacillus* sp. MTS (3 & 4)

Result of fermentation experiment implied that addition of nitrogen sources to the basal medium was important for keratinase production. Medium A was the basal medium, medium B was medium A + 0.02% triptone + 0.02% yeast extract and medium C was medium B + 0.02% glucose. Figure 2 showed that keratinase production of *Bacillus sp.* MTS was higher and faster than *B. licheniformis* MB-2. Maximum keratinolytic activity *B. licheniformis* MB-2 was obtained during stationery phase at 72 h. The highest activity were achieved in medium B (4.1 U/ml) and C (4.3 U/ml). While keratinase activity *Bacillus sp.* MTS was reached at 48 h and the highest activity were medium B (15.8 U/ml) and A (11.3 U/ml). Substrate induction was a major regulatory mechanism, and the keratinase biosynthesis was not completely repressed by addition of other carbon (glucose) and nitrogen (NH<sub>4</sub>Cl) sources (Ramnani and Gupta, 2004; Ignatova et al., 1999). The study on the supplementation of various carbon sources revealed that yeast extract supplementation resulted in increasing keratinolytic enzyme production. On the other hand glucose supplementation had an inhibitory effect on keratinolytic enzyme production (Yamamura et al., 2002). In our experiment, addition of 0.02% glucose to feather broth incubated with *Bacillus sp.* MTS resulted in decrease in keratinolytic activity (Figure 3). This catabolite repression by glucose is a well-known control mechanism for biosynthesis of microbial proteases including keratinases produced by *B. licheniformis* (Lin et al., 2001).

Based on the ability of *Bacillus sp.* MTS to produce extracellular keratinolytic enzymes and to degrade native-feather, we choosed this isolate to further keratinase production. We also observed the intracellular and extracellular keratinase activity produced by *Bacillus sp.* MTS. The extracellular keratinase obtained from free-cell culture, the cell washed three times with buffer and then lyzed the cell by sonication to obtain intracellular broth. Figure 3 showed the maximum keratinase activity obtained in extracellular broth at 48 h, while the intracellular disulfide reductase activity appeared to reach maximum activity at 24 h incubation. Complete degradation of the

hard keratin is possible only after the protein denaturation by splitting the disulphide bonds, because in general, the proteases of keratinolytic bacteria alone are unable to digest native hard keratin (Kunert, 2000). Protein in the extracellular environment or on the cell surface are usually rich in the disulfides, content to reflecting oxidizing conditions. In contrast, the inside of the cell is kept reduced and the protein usually contain many free sulphydryl group. Intracellular broth of *Bacillus* sp. is reported contain thioredoxin reductase, an enzyme catalyzes the reversible electron transfer from NADPH to oxidized thioredoxin. Reduced thioredoxin is the hydrogen donor for the enzyme ribonucleoside diphosphate reductase is needed for DNA synthesis (Arner and Holmgren, 2000).

*Bacillus sp.* MTS caused increase in the medium pH during cultivation on raw feather and was able to completely degradation native-feather. Organism with higher keratinolytic activity usually alkalinize the medium to a greater extent than those exhibiting lower keratinolytic activity (Riffel et al., 2003). Keratin degradation in a liquid medium is accompanied by the release of cleavage products into the cultivation fluid. Keratinolytic bacteria may accumulate high amounts of peptidic compounds in the medium. A better indicator of keratinolysis is the rise in pH medium, reflecting the utilization of keratin proteins, deamination and ammonia production. Figure 4 showed pH medium change at keratin degradation. Value of pH rise from 7.5 to 8.4, and the maximum keratinolytic enzymes activity attained at pH 8.0- 8.2.

The effect of temperature and pH on keratinase activity is showed in Figure 5. Activity was observed in the range of 25-95°C and pH 5.0-12.0. The maximum keratinase activity at 55°C and pH 8.0. This optimum of temperature and pH for keratinase activity was in good agreement with other known keratinolytic enzymes *Chryseobacterium* sp. (Riffel et al., 2003a); *Microbacterium* sp. (Thys et al., 2004); *Bacillus* sp. kr10 (Riffel et al., 2003b). *Bacillus* sp. strain kr16 had maximum keratinase activity at pH 8.0-11.0 and temperature 45-65°C (Werlang and Brandelli, 2005).

Figure 6 showed extracellular fluid *Bacillus* sp. MTS contains some protein bands. Three protein bands appear more clearer at second day incubation than at the first day incubation, this protein bands were 17 kDa, 25 kDa and 42 kDa approximately. This result was supported data at Figure 2 and 4 that the optimal extracellular keratinolytic activity of *Bacillus* sp. MTS was obtained at the second day of incubation.

The zymogram shown in Figure 7 indicates that sixth protein bands of 122, 96, 53, 32, 25, and 17 kDa catalyzed the hydrolysis of gelatin. Some bacteria produce protease keratinolytic

with various molecular weight. The molecular weight of protease keratinolytic *Thermoan aerobacter keratinophilus* is 135 kDa (Riessen and Antranikian, 2001), *Lysobacter* 148 kDa (Allpress et al., 2002) and *Bacillus* sp. 134 kDa (Lee et al., 2002). *Fervidobacterium islandicum* AW-1 produce homomultimer protease keratinolytic, it had molecular weight > 200 kDa (Nam et al., 2002), But some bacteria produce small protease keratinolytic, they are *Xanthomonas maltophilia* 36 kDa (Toni et al., 2002), *Thermoactinomyces candidus* 30 kDa (Ignatova et al., 1999) and *B. pumilus* 32 kDa (Huang et al., 2003).

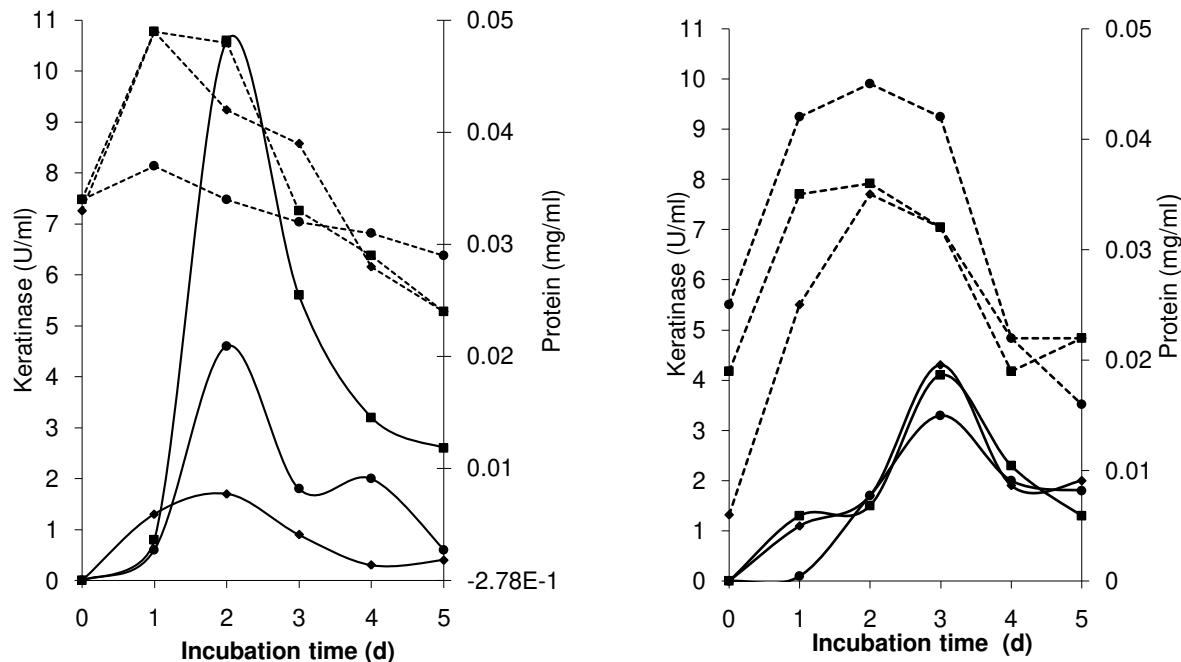


Figure 2. Activity of extracellular keratinase (solid line) by *B. licheniformis* MB-2 (right) and *Bacillus* sp. MTS (left) in feather meal medium. (-●- medium A -■- medium B -◆- medium C). The protein content is presented in dotted line (---)

Table 1. Nature keratin weight loss after degradation by *B. licheniformis* MB-2 and *Bacillus* sp. MTS

Native keratin	<i>B. licheniformis</i> MB-2 Weight loss (%)	<i>Bacillus</i> sp. MTS Weight loss (%)
Whole feather	10.96	27.1
Coccon	29.68	15.6
Silk	11.65	11.7
Human hair	7.50	10.1
Fish scales	61.30	54.1

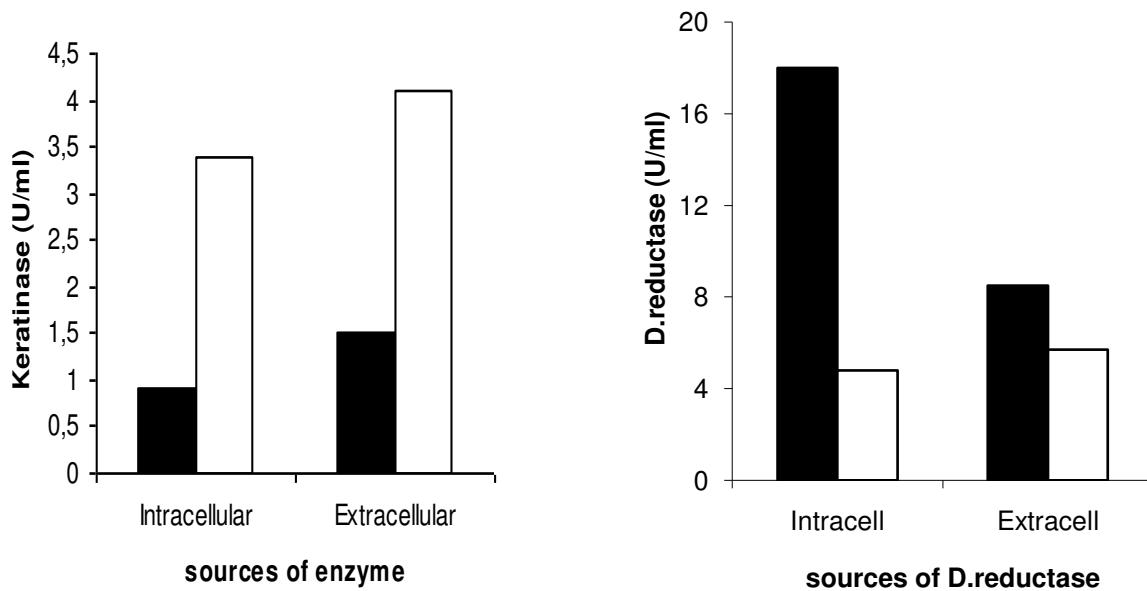


Figure 3. Keratinase and disulfide reductase activity of *Bacillus* sp. MTS of different enzymes preparation at 24 h (■) and 48 h (□) incubation

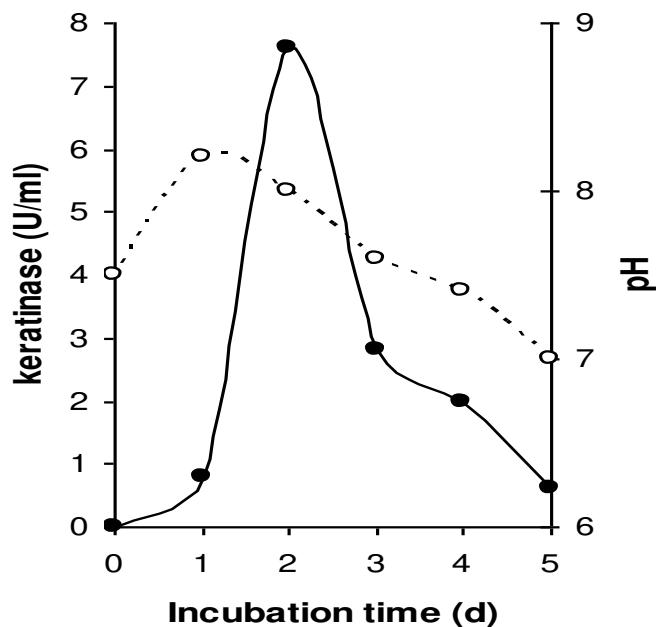


Figure 4. Activity of extracellular keratinase and the medium pHs during fermentation of *Bacillus* sp. MTS. Solid line (-) represent enzyme activity and dotted line (---) represent medium pH

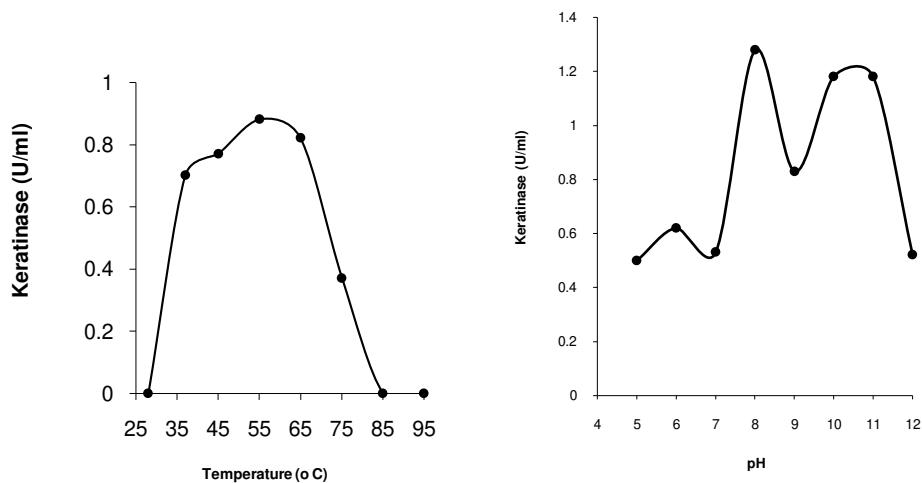


Figure 5. Effect of temperature and pH on keratinase activity of *Bacillus* sp. MTS. Keratinase activity was measured at different temperature and pH values

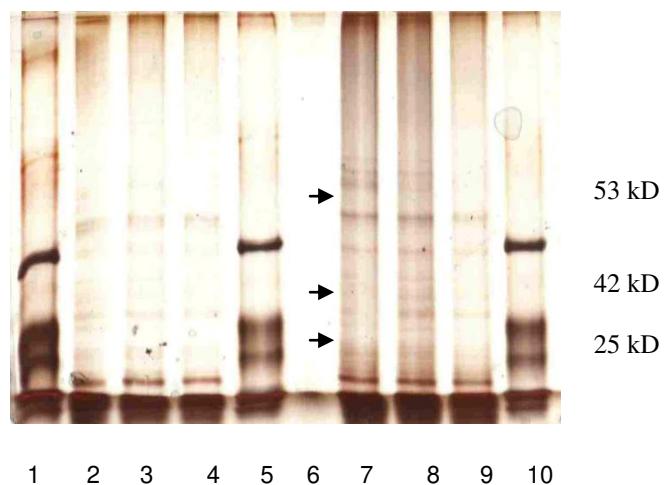


Figure 6. SDS-PAGE analysis of extracellular keratinase *Bacillus* sp. MTS at different time of incubation. Lane 1,5,10 : molecular weight markers. 2,3,4. : enzyme from 24 h incubation. 7,8,9. enzyme from 48 h day incubation

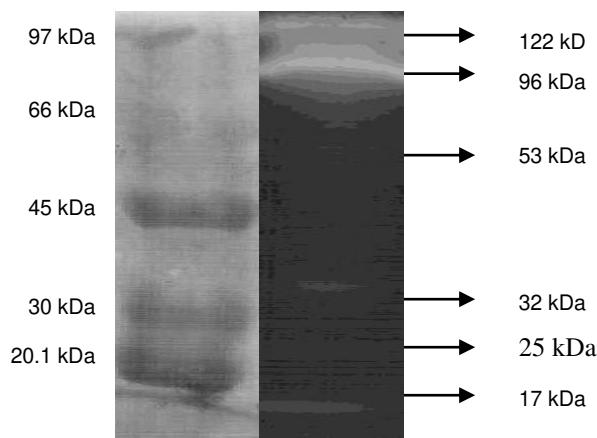


Figure 7. Zymograms of keratinase activity. Lane 1. molecular weight markers, 2. extracellular fluid *Bacillus* sp. MTS was precipitated with 65% (w/v) ammonium sulfate and dialysis

## Conclusions

*Bacillus sp.* MTS and *B. licheniformis* MB-2 were capable of degrading proteinous substrates rich in beta structure such as feather, cocoon, silk, human hair and fish scales. *Bacillus sp.* MTS was degraded whole feather more effective and efficient than *B. licheniformis* MB-2, its produce keratinase extracellular which active at 50°C and alkaline pHs. Addition 0.02% tryptone and 0.02% yeast extract was the best medium for keratinase production, and the maximum activity of keratinase obtained at 48 h of incubation. Zymogram analyzis of extracellular fluids *Bacillus sp.* MTS indicates that there were sixth protein bands catalyzed the hydrolysis of gelatin substrate in-situ.

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