African Journal of Biotechnology Vol. 8 (18), pp. 4590-4596, 15 September, 2009
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB09.545
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Isolation and characterization of a new keratinolytic bacterium that exhibits significant feather-degrading capability

Bo Xu[1,2,3#], Qiaofang Zhong[1,4#], Xianghua Tang[1,2,3], Yunjuan Yang[1,2,3] and Zunxi Huang[1,2,3]*

[1]School of Life Science, Yunnan Normal University, Kunming, 650092, P.R. China.
[2]Key Laboratory of Yunnan for Biomass Energy and Biotechnology of Environment, Yunnan, Kunming, 650092, P.R. China.
[3]Engineering Research Center of Sustainable Development and Utilization of Biomass Energy of Ministry of Education, Yunnan Normal University, Kunming, 650092, P.R. China.
[4]Institute of Biotechnology, Yunnan Agricultural Academy, China.

Accepted 11 June, 2009

A novel bacterium, *Bacillus licheniformis* K-19, which produces a large amount of a keratinase that is extremely thermostable and has a broad resistance to pH, was isolated and characterized. The maximum amount of keratinase activity (about 224 Uml^{-1}) was produced at 37°C when the bacterium was cultured for 72 h in broth containing feather meal with initial pH of 7.5. The keratinase activity was observed over a wide range of temperatures (30 - 90°C) and pH values (pH 6 - 10). It was optimal at 60°C and pH 7.5 - 8 respectively. These results suggest potential biotechnological applications of this bacterium that involve hydrolysis of keratin, including the improvement of the nutritional properties of feathers (and other keratins) used as supplementary feedstuffs.

Key words: *Bacillus licheniformis*, chicken feather, keratin, keratinolytic protease.

INTRODUCTION

Feathers are produced annually in huge amounts as waste products of commercial poultry-processing plants. Feathers are composed primarily of keratin. Because of a high degree of cross-linking by disulfide and other bonds, keratin is an insoluble protein and is not degraded by normal proteases such as trypsin, pepsin and papain. Although feathers contain large amounts of potentially useful protein and amino acids, they have limited use as dietary components in animal feedstuffs owing to their poor digestibility. Physical and chemical treatments are used currently to increase the digestibility of feather keratin. However, these processes require consumption of large amounts of energy and they also destroy certain amino acids, thus yielding products of poor digestibility and variable nutrient quality. Dymatic hydrolysis by microorganisms that possess keratinolytic activity represents an attractive alternative to improve the nutritional value of feather wastes.

Keratinases are enzymes that hydrolyzing keratin specifically. Keratinolytic enzymes may have potential roles in biotechnological processes that involving keratin-containing wastes from the poultry and leather industries.

Keratinolytic activity has been reported for various bacterial genera, such as *Bacillus* (Williams et al., 1990; Lin et al., 1999; Manczinger et al., 2003; Suntornsuk and Suntornsuk, 2003; Zerdani et al., 2004; Suntornsuk et al., 2005), *Thermoanaerobacter* (Riessen and Antranikian, 2001), *Chryseobacterium* (Riffel et al., 2003), *Flavobacterium* (Riffel and Brandelli, 2002; Nam et al., 2002) and *Vibrio* (Sangali and Brandelli, 2000). The keratinase of *B. licheniformis* PWD-1, which was isolated from a poultry waste digester, and the gene (*kerA*) that encodes this keratinase have been isolated and characterized (Williams *et al*. 1990, Lin et al., 1992, Cheng *et al*. 1995, Lin et al., 1995). This keratinase is a serine protease and has high homology with subtilisin Carlsberg from *B. subtilis* (Evans et al., 2000). Strains

*Corresponding author E-mail:huangzunxi@163.com. Fax: 86-0871-5847822.

#These authors contributed equally to this article

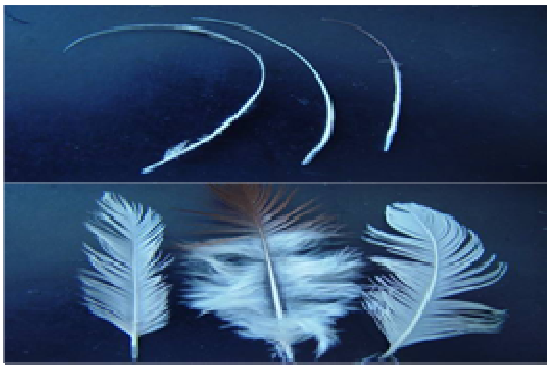

Figure 1. All feather barbules and almost all feather raches were degraded by keratinolytic bacterium K-19 after 2 - 3 days.

L-25 and FK14 of *B. licheniformis*, which demonstrate effective keratinolytic activity, were isolated from canola meal compost and Thai soil respectively (Lin et al., 1999; Suntornsuk et al., 2005).

In this article, we report the isolation and characterization of a new bacterium, *B. licheniformis* K-19, which can digest native chicken feathers completely within 2 - 3 days. The properties of the crude keratinase obtained from this strain were also characterized.

MATERIALS AND METHODS

Isolation of keratinolytic microorganism

Samples were collected from several sites (e.g., the waste heap, sewage, and sludge) in which feathers were deposited in Kunming, Yunnan Province, P R China in September, 1997. These samples were enriched in broth containing feather meal (NH_4Cl, 0.5; NaCl, 0.5; K_2HPO_4, 0.3; KH_2PO_4, 0.4; $MgCl_2{\cdot}6H_2O$, 0.1; feather powder, 10.0 g l^{-1}; pH 7.5), in which feathers served as the sole source of carbon, nitrogen, sulfur and energy. The cultures were incubated at 37°C with stirring at 180 rpm for 2-5 days. The samples that showing the greatest degradation of feathers were loaded on to milk-agar plates (NH_4Cl, 0.5; NaCl, 0.5; K_2HPO_4, 0.3; KH_2PO_4, 0.4; Mg $Cl_2{\cdot}6H_2O$, 0.24; yeast extract 2.0; evaporated whole milk 20.0; agar 20.0 g l^{-1}; pH 7.5) for primary screening. Protease-producing bacteria that formed clear haloes were selected. They were transferred subsequently at frequent intervals to feather meal broth for adaptation to keratin degradation. The isolates that caused complete disintegration of whole feathers were chosen for further analysis.

Growth determination

An isolate from a 10^6 CFU ml^{-1} culture was cultivated initially at pH 7.5 for 72 h in feather meal medium (NH_4Cl, 0.5; NaCl, 0.5; K_2HPO_4, 0.3; KH_2PO_4, 0.4; $MgCl_2{\cdot}6H_2O$, 0.24; $CaCl_2$, 0.1; yeast extract, 2.0; feather meal, 10.0 g l^{-1}). Bacterial growth was estimated by direct measurement of the CFU ml^{-1} (Sangali and Brandelli, 2000).

Assays of enzyme activity

The organism was cultivated in the basal feather powder broth at 37°C and 180 rpm for 72 h. The bacterial suspension (approximately 1×10^6 CFU ml^{-1}) was used as an inoculum (2% v/v). The crude enzyme was prepared by centrifugation at 10,000×*g* for 10 min.

Keratinolytic activity was measured using insoluble azokeratin as a substrate (Lin et al., 1992). Azokeratin was synthesized based on the methodology described for azoalbumin (Tomarelli et al., 1949).

Taxonomic studies

Bacteria were identified based on morphological, physiological and biochemical tests (Claus and Berkeley, 1986) and analysis of 16S rDNA sequences. Genomic DNA was extracted from isolate K-19 in LB broth at 37°C for 24 h. The 16S rDNA gene was amplified by PCR using primers 8f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1523r (5′-AAGGAGGTGATCCAGCCGCA-3′), followed by sequencing. The BLAST program was used to search for homologous sequences in the GenBank, EMBL and DDBJ databases. The 16S rDNA sequences were aligned and compared with similar database sequences using the Clustal X program.

Characterization of the keratinolytic protease

Characteristics of the crude enzyme were studied using an assay of keratinolytic activity. The influence of temperature on keratinase activity was determined between 30 and 90°C. In order to investigate the thermostability of the keratinase, the diluted enzyme solution was pre-incubated for 10-90 min at 50-70°C, followed by measurement of the residual activity according to a standard assay method. The effect of pH on keratinolytic activity was determined between pH 4 and 11. In order to determine pH stability, the enzyme solution was pre-incubated in each buffer at room temperature for 1 h, and the residual activity was then determined using the standard enzyme assay.

To investigate the effects of metal ions and other chemical reagents on keratinase activity, the activity was tested after pre-incubation of the enzyme solution with each compound for 30 min at room temperature.

Determination of protein concentration

The cell-free supernatant fluid from each feather culture was used for the measurement of soluble protein using the Folin phenol reagent method with bovine serum albumin as standard.

RESULTS

Isolation of keratinase-producing strains

From enrichment cultures, a total of 36 strains capable of degrading milk protein underwent primary screening on milk-agar plates. Among them, isolate K-19 demonstrated the highest feather-degrading activity and hydrolyzed completely native chicken feathers at 37°C within 3-4 days. All feather barbules and almost all feather raches were degraded after 2 - 3 days (Figure 1).

Production of keratinase and soluble protein

Keratinase activity and growth of the isolate K-19 was followed during cultivation in the basal broth containing

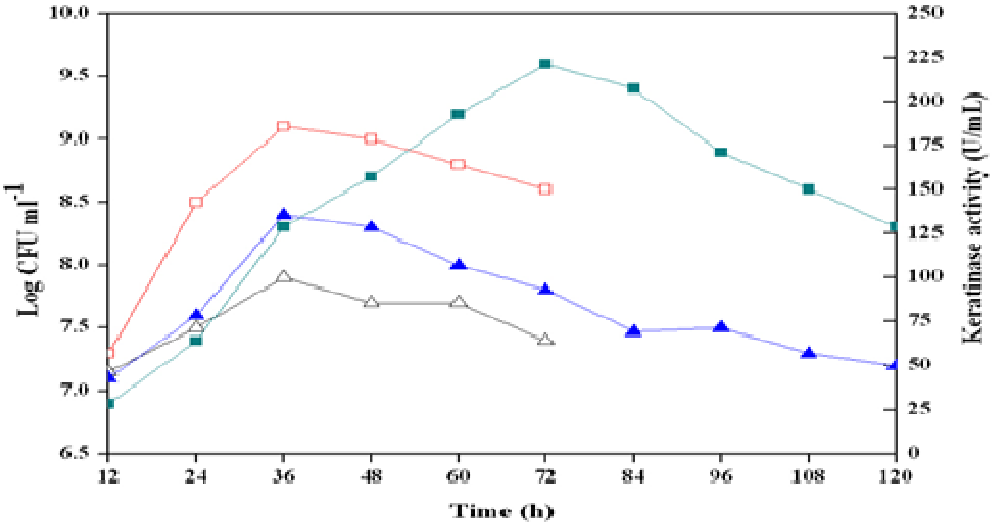

Figure 2. Growth and keratinase activity of the isolate K-19 during cultivation in basal feather meal broth at 37°C, 50°C and initial pH 7.5. Bacterial growth at 37°C (□) and 50°C (△) was estimated by direct measurement of the CFU ml^{-1}. Keratinolytic activity at 37°C (■) and 50°C (▲) was measured using insoluble azokeratin as a substrate under standard assay conditions. Points represent the means of three independent experiments.

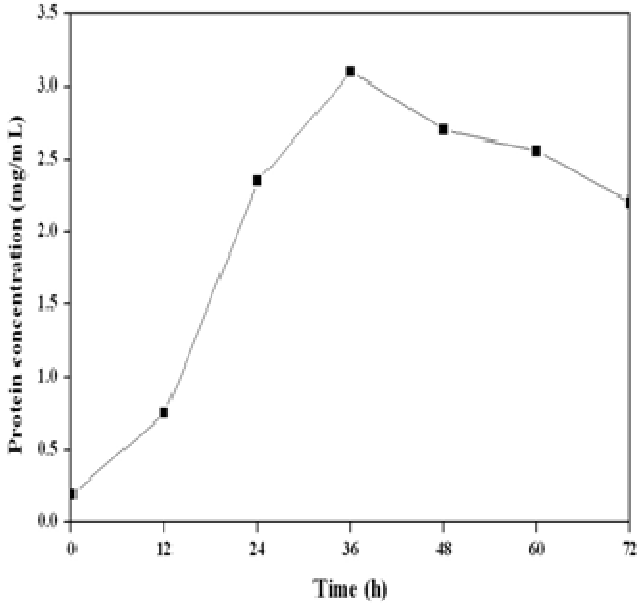

Figure 3. The concentration of soluble protein during cultivation in feather powder broth at 37°C. Cell-free supernatant fluid from each feather culture was used for the measurement of soluble protein by the Folin phenol reagent method with bovine serum albumin as standard. Each point represents the mean of three independent experiments.

feathers at 37 and 50°C and initial pH 7.5. The growth and keratinase production were optimal at 37°C (Figure 2). The highest level of keratinase activity (about 224 Uml^{-1}) was produced at 72 h. An increase in the pH of the medium to above 8.0 (about 8.1 - 8.8) was notable after 2 -3 days of cultivation.

This is a typical phenomenon that accompanies the hydrolysis of keratin and demonstrates the keratinolytic potential of microorganisms. The amount of protein released into the culture medium as a result of the degradation of feather keratin was determined. The maximum concentration of soluble protein (approximately 3.0 mg/ml) was obtained at 37°C during 36 h of cultivation (Figure 3). Strains that possess high keratinase activity release large amounts of protein into the culture medium.

Characterization of strain K-19

The isolate K-19 proved to be a Gram-positive bacterium with straight rod-shaped cells. It possessed a capsule and one central or subterminal oval endospore per cell. It formed opaque pink colonies on milk-agar plates; the colonies were irregular in shape, moist and rough. The optimal temperature for growth was 37°C. No growth occurred below 20°C or above 60°C. The isolated strain grew at pH 5.0 - 11.0 with an optimal pH of 7-8.

Together with the physiological and biochemical characteristics, these results indicate that K-19 belongs to the genus *Bacillus*. Analysis of the 16S rDNA sequence of K-19 revealed that this strain showed the highest homology (more than 99%) with *Bacillus licheniformis* strains. From these results, K-19 was identified as a new isolate of the species *B. licheniformis*. The 1,502-bp 16S rDNA gene sequence of K-19 has been submitted to the Genbank database under the accession number DQ35 1932.

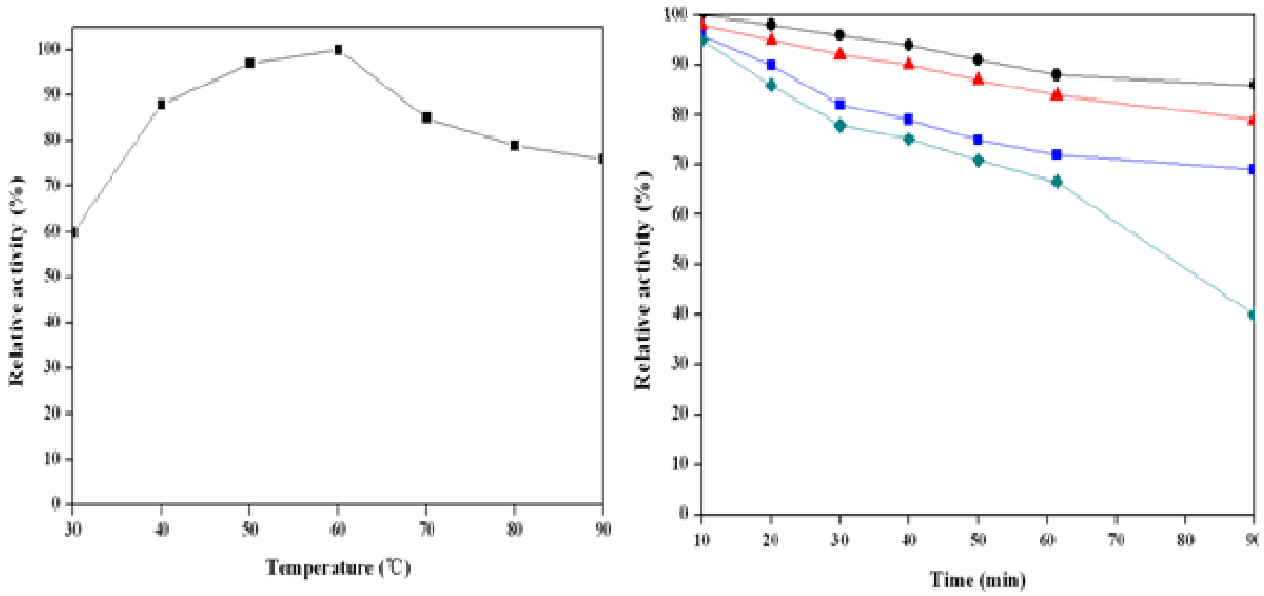

Figure 4. Effect of different temperatures on keratinase activity (A) and thermostability (B). The effect of temperature on keratinolytic activity was investigated at different temperatures (30–90℃) in 50mM glycine/NaOH buffer (pH 8). For investigation of temperature stability, the diluted enzyme solution was pre-incubated at various temperatures, 50℃ (●), 60℃ (▲), 65℃ (■) and 70℃ (◆), for different lengths of time (10–90 min). The relative keratinolytic activity was recorded with reference to an assay run at 50℃ in glycine/NaOH buffer (pH 8) = 224U/ml. Each point represents the mean of three independent experiments.

Preliminary characterization of the keratinase

Enzyme activity was observed in the range 30-90℃, with maximal activity at 60℃, and was reduced rapidly at higher temperatures (Figure 4A). The keratinase activity was stable at 50-60℃, with more than 80% of the initial activity remaining after 90 min. At 65 and 70℃, the keratinase activity was relatively thermostable, with approximately 80% of the initial activity remaining after 40 min and about 70% residual activity after 90 min of incubation at 65℃ (Figure 4B). The keratinase was active in the pH range 6-10 with optimal activity at pH 7.5 - 8. It was also stable over a wide range of pH (6-10), with the highest stability at pH 7-8.5 (Figure 5).

Tables 1 and 2 summarize the effects of metal ions and other chemical reagents on the activity of the keratinase. The enzyme was partially inhibited by EDTA (62.5%), 1, 10-phenanthroline (56.5%) and SDS (77.8%) (Table 1). The enzyme was stable with detergents such as Tween 20 and Tween 80 and organic solvents such as dimethyl sulfoxide, methanol and isopropanol. The reducing agent β-mercaptoethanol caused a significant increase in keratinolytic activity. The enzyme was wholly inhibited by Hg^{2+} and Ag^{+} (Table 2).The activity of the enzyme was partially inhibited by Fe^{2+}, Ni^{2+}, Cu^{2+}, Ba^{2+}, and Al^{3+}. However, the activity was slightly enhanced in the presence of K^{+}, Mn^{2+}, Na^{+}, and Ca^{2+}. Exposure to Zn^{2+} and Cd^{2+} caused significant enhancement of the keratinase activity. The enzyme was influenced slightly by Mg^{2+}, Fe^{3+}, and Co^{2+}.

DISCUSSION

The microbial conversion of feather wastes is a potential technique for the degradation of feathers and their utilization as a feedstuff (Sangali and Brandelli, 2000; Riffel et al., 2003). Microorganisms that degrade feathers and their keratinolytic enzymes could be used to enhance the digestibility of feather keratin (Suntornsuk and Suntornsuk, 2003; Zerdani et al., 2004; Suntornsuk et al., 2005).

Many *Bacillus* species have been reported to produce keratinolytic proteases (Lin et al., 1992; Cheng et al., 1995; Lin et al., 1999; Manczinger et al., 2003; Suntornsuk and Suntornsuk, 2003; Zerdani et al., 2004; Suntornsuk et al., 2005). The most effective keratin-degrading strains in the *Bacillus* genus belong to the species *B. licheniformis*. Table 3 summarizes the keratinolytic activity and biochemical properties of the keratinases of K-19 and other strains of *B. licheniformis*.

According to previous studies, *B. licheniformis* PWD-1 degrades native feathers completely when cultured at 50℃ for 10 days, and presents optimum growth and keratinase production at 50℃ (Williams et al. 1990, Lin et al. 1992, Cheng et al. 1995). The keratinase from *B. licheniformis* PWD-1 showed high keratinolytic activity that was optimal at pH 7.5 and 50 - 55℃. However, secretion of this enzyme was suppressed totally in the presence of glucose and its activity was lost entirely at elevated temperatures (above 65℃), probably as a result of enzymatic autolysis. These features are not compatible with the

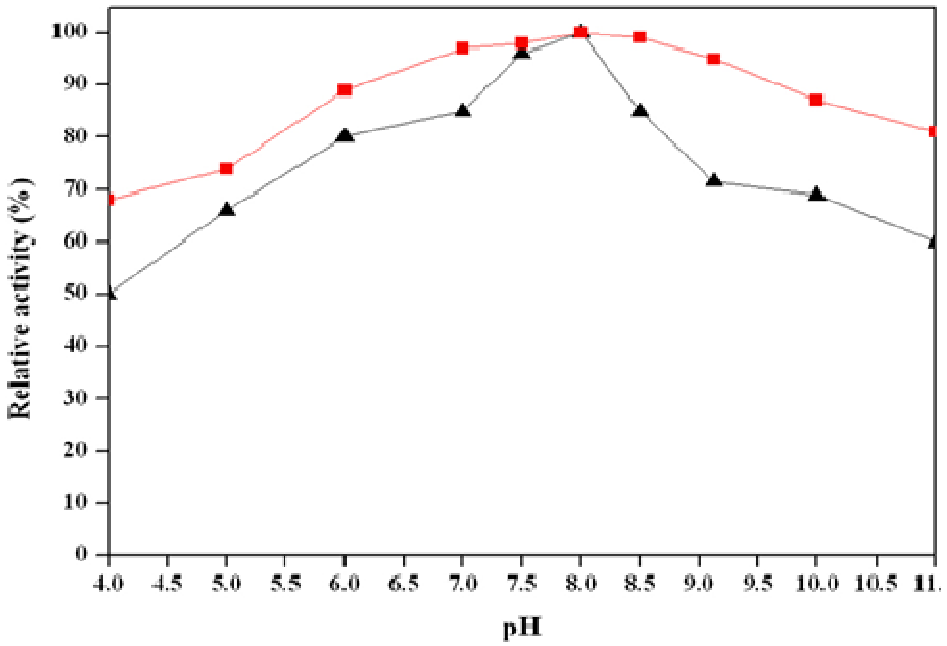

Figure 5. Effect of pH on the activity and stability of the keratinase. The enzyme assay was performed at 60°C in buffers at various values of pH (pH 4–11). For investigation of pH stability, the enzyme solution was pre-incubated at room temperature in buffers of pH 4 - 11 for 1 h, and the residual activity was then determined by the standard procedure. The buffers (50 mM) used contained: phosphate/citric acid buffer (pH 4 - 6), potassium phosphate buffer (pH 6 - 8), glycine/NaOH buffer (pH 8 - 10) and sodium bicarbonate buffer (pH 9 - 11). The relative keratinolytic activity was taken with reference to the reading of an assay run at 60°C in glycine/NaOH buffer (pH 8) = 224U/ml. ▲, Optimal pH; ■, pH stability. Each point represents the mean of three independent experiments.

Table 1. The effect of chemical reagents on the keratinase activity of *B. licheniformis* K-19.

Substance group	Compound	Concentration	Relative activity (%)
Control			100
Detergents	Triton X-100	0.5%(v/v)	87.2
		1% (v/v)	80.7
	SDS	0.5%(w/v)	77.8
	Tween 20	1%(v/v)	91.6
	Tween 80	1%(v/v)	91.8
Solvents	Dimethyl sulfoxide	1%(v/v)	99.8
		5%(v/v)	95.5
	Isopropanol	1%(v/v)	94.4
		5%(v/v)	92.8
	Methanol	1%(v/v)	92.6
Reducing agent	β–Mercaptoethanol	0.1%(v/v)	129.4
		0.5%(v/v)	133.8
Inhibitors	EDTA	5mM	62.5
	1,10-phenanthroline	1mM	56.5

The chemicals were added to the enzyme preparations and incubated for 30 min at room temperature before being tested for keratinase activity according to the working concentrations described in the Materials and methods.
Values are the means of three independent measurements. The keratinase activity of the enzyme solution without addition of any compound was taken as 100%.

Table 2. The effect of metal ions on the keratinase activity of *B. licheniformis* K-19.

Metal ion	Relative activity (%)	Metal ion	Relative activity (%)
None	100		
K^+	116.3	Ni^{2+}	75
Na^+	112.7	Zn^{2+}	124.8
Mg^{2+}	96.5	Ba^{2+}	86.7
Ca^{2+}	102.5	Hg^{2+}	0
Mn^{2+}	109.6	Ag^+	0
Fe^{3+}	93.9	Al^{3+}	86.4
Fe^{2+}	71.6	Co^{2+}	96.6
Cu^{2+}	63.6	Cd^{2+}	122.3

The metal ions tested were added at 10 mM.
Values represent the means of three independent measurements. The keratinase activity of the enzyme solution without addition of any metal ion was taken as 100%.

Table 3. Comparison of the keratinolytic activity and biochemical properties of the K-19 keratinase and keratinases from other strains of *B. licheniformis*.

Origin	OTkp[a] (℃)	Opkp[b]	OTkr[c] (℃)	Opkr[d]	Mka[e] (U/ml)	Reference
*B.licheniformis*PWD-1	50 - 55	8.7	50	7.5	3.5U/ml[f], 48h	Cheng et al. 1995
B.licheniformis L-25	40 - 45	-	-	-	11.1U/ml[f],60h	Lin et al. 1999
*B.licheniformis*FK14	50	-	60	8.5	3.67U/ml[g], 72h	Suntornsuk et al. 2005
*B.licheniformis*K-508	47	7	52	8.5	224U/ml[f], 72h	Rozs et al. 2001
*B.licheniformis*K-19	37	7 - 8	60	7.5 - 8		This study

[a] Optimal growth temperature for keratinase production in feather powder broth.
[b] Optimal initial pH for keratinase production in feather powder broth.
[c] Optimal temperature for keratinase reaction.
[d] Optimal pH for keratinase reaction.
[e] Maximum keratinase activity in feather powder broth.
[f] The keratinase activity was measured using insoluble azokeratin as a substrate. One unit of keratinolytic activity was defined as an increase of 0.01 absorbance unit at 450nm after reaction for 15 min under standard assay conditions.
[g] The keratinase activity was measured using the insoluble keratin azure assay. One unit of keratinolytic activity was defined as an increase of 0.1 absorbance unit at 595nm after reaction for 1 h under standard assay conditions.

practical commercial application of this enzyme. Strain L-25 of *B. licheniformis* is capable of hydrolyzing feathers effectively and produced its highest keratinase activity (11.1 Uml^{-1}) at 40-45℃ when incubated for 60 h (Lin et al. 1999), but its keratinase activity was apparently lower than that of other known *Bacillus* keratinolytic proteases. A new strain of *B. licheniformis*, K-508, has also been shown to degrade chicken feathers efficiently at pH 7.0 and 47℃, and its optimal pH and temperature were 8.5 and 52℃. However, these properties are also not more suited to practical commercial applications than those of the keratinolytic proteases of other *Bacillus* strains (Rozs et al., 2001). Although *B. licheniformis* FK14 presented optimal keratinolytic activity at pH 8.5 and 60℃, the keratinase of FK14 exhibited poor thermostability at 50-60℃, with only 40% of the initial activity remaining after 60 min (Suntornsuk et al. 2005). These results suggest that a number of keratinases from strains previously studied are not suitable for commercial applications of keratinolysis.

In our investigation, a new keratinase-producing bacterium (K-19) was isolated from the sewage waste at a local poultry farm. Complete feather degradation was achieved within only 2–3 days. An increase in pH was a significant indicator of its strong keratinolytic activity during cultivation. Organisms with higher keratinase activity turn the media more alkaline than those showing lower keratinolytic ability. This may be due to the deamination of peptides and amino acids that are produced by the degradation of keratin. On the basis of its morphological and biochemical characteristics, and 16S rDNA sequence analysis, this strain was identified as *B. licheniformis*. *B. licheniformis* K-19 produced its maximum amount of keratinase activity (224 Uml^{-1}) at 37℃ when incubated for 72 h in feather meal broth. The keratinase activity was observed over a wide range of temperature (30-90℃) and pH (pH 6-10), and was optimal at 60℃ and at pH 7.5-8. In addition, the keratinase was stable over a wide range of

pH (6-10) and exhibited excellent thermostablity at 50-60°C, with over 80% of the initial activity remaining after 90 min. The keratinase activity was also relatively stable at 65 and 70°C, with approximately 80% of the initial activity remaining after 40 min.

From the above results, we conclude that *B. licheniformis* K-19 can secrete a large amount of a keratinase that is more thermostable and has broader pH resistance than other keratinolytic proteases from *Bacillus* reported previously (Lin et al., 1992; Cheng et al., 1995; Lin et al., 1999; Suntornsuk et al., 2003; Suntornsuk et al., 2005). The keratinase from this strain degraded feathers effectively and possessed interesting and potentially useful properties. Therefore, the newly isolated *B. licheniformis* K-19 shows potential for use in biotechnological processes that involve keratin hydrolysis, and it can be expected to improve the nutritional value of animal feeds that contain feathers (and other keratins) or wastes from poultry processing.

ACKNOWLEDGEMENTS

Financial support was provided by the key project of Yunnan basic applied plan (Grant No.2006C0004Z) and the professional project of Yunnan key Industry technology, High technology Dept. of Yunnan Province development and reform commission (Grant No.20081657), National high-technology Development Project (Grant No.2008AA02Z202, No.2007AA02Z217).

REFERENCES

Cheng SW, Hu HM, Shen SW, Takagi H, Asano M, Tsai YC (1995). Production and characterization of keratinase of a feather-degrading *Bacillus licheniformis* PWD-1. Biosci Biotechnol. Biochem. 59: 2239-2243.

Claus D, Berkeley RCW (1986). Genus *Bacillus*. In Bergey's Manual of Systematic Bacteriology, Volume2, eds. Sneath PHA, Mair NS, Sharpe ME. pp. 1105-1139. Baltimore: Williams, Wilkins. ISBN 0-683-07893-3.

Evans KL, Crowder J, Miller ES (2000). Subtilisins of *Bacillus* spp. hydrolyze keratin and allow growth on feathers. Can. J. Microbiol. 6: 1004-1011.

Lin X, Lee CG, Casale ES, Shih JCH (1992). Purification and characterization of a keratinase from a feather- degrading *Bacillus licheniformis* strain. Appl. Environ. Microbiol. 58: 3271- 3275.

Lin X, Kelemen DW, Miller ES, Shih JCH (1995). Nucleotide sequence and expression of kerA, the gene encoding a keratinolytic protease of *Bacillus licheniformis* PWD-1. Appl. Environ. Microbiol. 61: 1469-1474.

Lin X, Inglis GD, Yanke LJ, Cheng KJ (1999). Selection and characterization of feather-degrading bacteria from canola meal compost. J. Ind. Microbiol. Biotechnol. 23: 149-153.

Manczinger L, Rozs M, Va′gvo¨lgyi Cs, Kevei F (2003). Isolation and characterization of a new keratinolytic *Bacillus licheniformis* strain. World J. Microbiol. Biotechnol. 19: 35-39.

Nam GW, Lee DW, Lee HS, Lee NJ, Kim BC, Choe EA, Hwang JK, Suhartono MT, Pyun YR (2002). Native-feather degradation by *Fervidobacterium islandicum* AW-1, a newly isolated keratinase-producing thermophilic anaerobe. Arch Microbiol. 178: 538- 547.

Riessen S, Antranikian G (2001). Isolation of *Thermoanaerobacter keratinophilus* sp. nov., a novel thermophilic, anaerobic bacterium with keratinolytic activity. Extremophiles, 5: 399-408.

Riffel A, Brandelli A (2002). Isolation and characterization of a feather-degrading bacterium from the poultry processing industry. J. Ind. Microbiol. Biotechnol. 29: 255-258.

Riffel A, Lucas F, Heeb P, Brandelli A (2003). Characterization of a new keratinolytic bacterium that completely degrades native feather keratin. Arch Microbiol. 179: 258-265.

Rozs M, Manczinger L, Vágvölgyi C, Kevei F (2001). Secretion of a trypsin- like thiol protease by a new keratinolytic strain of *Bacillus licheniformis*. FEMS Microbiol. lett. 205: 221-224.

Sangali S, Brandelli A (2000). Feather keratin hydrolysis by a *Vibrio* sp. strain kr2. J. Appl. Microbiol. 89: 735-743.

Suntornsuk W, Suntornsuk L (2003). Feather degradation by *Bacillus* sp. FK 46 in submerged cultivation. Bioresour. Technol. 86: 239-243.

Suntornsuk W, Tongjun J, Onnim P, Oyama H, Ratanakanokchai K, Kusamran T, Oda K (2005). Purification and characterisation of keratinase from a thermotolerant feather-degrading bacterium. J. Ind. Microbiol. Biotechnol. 21: 1111-1117.

Tomarelli RM, Charney J, Harding ML (1949). The use of azoalbumin as a substrate in the colorimetric determination of peptic and tryptic activity. J. Lab. Clin. Med. 34: 428-433.

Williams CM, Richester CS, Mackenzi JM, Shih JCH (1990). Isolation, identification and characterization of a feather-degrading bacterium. Appl. Environ. Microbiol. 56: 1509-1515.

Zerdani I, Faid M, Malki A (2004). Feather wastes digestion by new isolated strains *Bacillus* sp. in Morocco. Afr. J. Biotech. 3: 67-70.