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Keratinase production and keratin degradation by a mutant strain of *Bacillus subtilis**

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Abstract: A new feather-degrading bacterium was isolated from a local feather waste site and identified as *Bacillus subtilis* based on morphological, physiochemical, and phylogenetic characteristics. Screening for mutants with elevated keratinolytic activity using *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine mutagenesis resulted in a mutant strain KD-N2 producing keratinolytic activity about 2.5 times that of the wild-type strain. The mutant strain produced inducible keratinase in different substrates of feathers, hair, wool and silk under submerged cultivation. Scanning electron microscopy studies showed the degradation of feathers, hair and silk by the keratinase. The optimal conditions for keratinase production include initial pH of 7.5, inoculum size of 2% (v/v), age of inoculum of 16 h, and cultivation at 23 °C. The maximum keratinolytic activity of KD-N2 was achieved after 30 h. Essential amino acids like threonine, valine, methionine as well as ammonia were produced when feathers were used as substrates. Strain KD-N2, therefore, shows great promise of finding potential applications in keratin hydrolysis and keratinase production.

Key words: *Bacillus subtilis*, Keratin, Keratin degradation, Keratinase production, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) mutagenesis

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INTRODUCTION

Feathers are composed of over 90% protein and produced in large amounts as a waste by poultry processing worldwide. Accumulation of feathers will lead to environmental pollution and feather protein wastage (Onifade *et al*., 1998; Gousterova *et al*., 2005). Traditional ways to degrade feathers such as alkali hydrolysis and steam pressure cooking may not only destroy the amino acids but also consume large amounts of energy. Biodegradation of feathers by keratinase from microorganisms may provide a viable alternative. *Bacillus* (Williams *et al*., 1990; Riffel *et al*., 2003; Manczinger *et al*., 2003; El-Refai *et al*., 2005), fungi (Gradišar *et al*., 2000; Friedrich *et al*., 2005) and *Actinomycetes* (Ignatova *et al*., 1999; Gousterova *et al*., 2005) have previously been shown to be able to produce feather-degrading keratinases.

Keratinase and related products have many applications (Gupta and Ramnani, 2006). For example, the feather hydrolysates of *Bacillus licheniformis* PWD-1 and *Vibrio* sp. strain kr2 (Williams *et al*., 1991; Grazziotin *et al*., 2006) can be used as feed additives, while the keratinase from *Bacillus subtilis* S14 exhibits remarkable dehairing capabilities (Macedo *et al*., 2005). Moreover, keratinase from *B. licheniformis* PWD-1 can degrade the infectious form of prion, PrP^{sc}, in the presence of detergents and heat treatment (Langeveld *et al*., 2003), which is very important for the utilization of animal meal as feed. Usually, it is important to improve the enzyme yield for application purposes and so various methods including the optimization of cultural conditions and medium composition, or heterologous gene expression have been applied (Ramnani and Gupta, 2004; Anbu *et al*., 2005).

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Given the effectiveness of traditional mutagenesis approach for isolating mutants that produce improved yields of various microbial enzymes such as lipase and α-galactosidase (Tan *et al*., 2003; Wang *et al*., 2004), it is conceivable that a similar strategy may be successfully applied to improve the ability of keratinase-producing strains for the production of this important enzyme.

Almost all keratinases are inducible and different keratin-containing materials such as feathers, hair and wool can be used as substrates for keratinase production (Gupta and Ramnani, 2006). Feather was the mostly utilized substrate, while human hair was rarely utilized, especially by *Bacillus* sp. Another keratin-containing materials, silk as well as feathers and hair, are largely produced in China and these may also be potential substrates for keratinase production.

The aim of this study was to identify a newly isolated feather-degrading bacterium strain, to characterize keratinase production and keratin degradation in feathers, hair and silk by a *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) mutagenesis strain with improved keratinolytic activity, and to optimize the conditions for keratinase production in feather substrates.

MATERIALS AND METHODS

Culture media

The basic medium used for isolation and fermentation of the feather-degrading microorganisms contained the following constituents (g/L): NaCl (0.5), KH_2PO_4 (0.7), K_2HPO_4 (1.4), $MgSO_4$ (0.1) and feathers (10), pH 7.2. Cultivation was done using 500 ml Erlenmeyer flasks containing 100 ml medium. Feather agar medium containing the basic medium and 20 g/L of agar was used for screening the microorganisms in plates. For the medium used for screening mutants, 10 g casein was used instead of feathers. Luria-Bertani (LB) medium (peptone 1% (w/v), yeast extract 0.3% (w/v), NaCl 0.5% (w/v), pH 7.2) was used for inoculum preparation and isolate maintenance.

Isolation of keratinolytic microorganisms

Rotted feather samples and soil were collected from a local poultry plant. The samples were shaken in 0.85% (w/v) NaCl solution for 30 min, and 100 μl of the suspension was plated on feather agar plates, followed by cultivation at 37 °C for 48 h. Well-grown single colonies were isolated and purified by streak-plating onto new feather agar plates. The largest single colony on the plate was isolated and inoculated into feather medium-containing flasks and shaken at 37 °C for 3 d. The feathers in flasks were degraded intensively by the purified isolate, designated as KD-1 and maintained on LB slants at 4 °C for further work.

Mutagenesis and screening

Strain KD-1 was cultivated in LB medium at 37 °C for 20 h, followed by centrifuging 10 ml of the cells at 1450×g for 15 min. The cell pellet was diluted in 0.1 mol/L sterile phosphate buffer (pH 7.2) and adjusted to a concentration of 10^6 CFU/ml; then 1 ml of the cell suspension was incubated with 1 ml 1 mg/ml MNNG solution (in phosphate buffer, pH 7.2) at 30 °C for different periods of time (10~60 min, with 10 min increments). Finally the reactions were stopped, 100 μl serially diluted aliquots were plated on casein plates and cultivated at 37 °C for 48 h. For determination of the keratinolytic activity of the wild-type and two mutants, flask cultivation was carried out at 37 °C and 200 r/min for 30 h.

Taxonomical studies

Morphological studies were conducted using light and electron microscopy (XL30-ESEM environment scanning electron microscopy, Philips, the Netherlands), characteristics of the isolate were compared with data from *Bergey's Manual of Systematic Bacteriology* (Liu, 1984).

Carbohydrate metabolism tests were performed by the API 50 strips (bioMérieux, Lyon, France) and the resultant emerging biochemical profiles were identified by the APILAB software version ATB278c, 2000 (bioMérieux, Lyon, France).

Genomic DNA from the strain KD-1 was isolated as described by Sambrook *et al*.(1989). The 16S rDNA gene was amplified by PCR using primers 5′-GCG TGC CTA ATA CAT GCA AG-3′ and 5′-AAG GTT ACC TCA CCG ACT TC-3′ designed from the conserved sequences of *B*. *subtilis* strains. The amplified PCR product of 1360 bp was sequenced and submitted to GenBank (Acession No. DQ504376).

BLAST algorithm was used to search for homologous sequences in GenBank. The 16S rDNA sequences were aligned using the ClustalX program (Thompson *et al*., 1997) and the phylogenetic tree was booted by the MEGA3 software (Kumar *et al*., 2004).

Effects of substrates on keratinase production

One gram of skim milk powder, casein, hair, peptone, wool and silk as well as 0.1, 0.5, 1.0, 1.5 and 2.0 g feathers were used as sole sources of carbon and nitrogen source for keratinase production. Cultivation was performed at 200 r/min and 37 °C for 24 h except for hair, wool or silk, where the cultivation time was extended to 72 h.

Effects of cultural conditions on keratinase production and residual hydrolysates

For optimization, production of keratinase by KD-N2 was studied using 1 g feather substrate under the following conditions: initial pH 6.5~8.5 with increments of 0.5 unit, temperatures 18~42 °C with increments of 4 or 5 °C, inoculum size 2%~10% (v/v) of cell density 10^7 CFU/ml, age of inoculum 12 to 24 h with increments of 4 h. Five-hundred millilitres Erlenmeyer flasks containing 100 ml culture medium were incubated at 28 °C and 200 r/min for 24 h. The residual hydrolysates were removed by centrifugation at 1450×g for 30 min and dried for their determination. The cell free supernatant was analyzed for keratinase activity.

Preparation of keratin solution

Keratinolytic activity was measured with soluble keratin (0.5%, w/v) as substrate. Soluble keratin was prepared from white chicken feathers by the method of Wawrzkiewicz *et al*.(1987). Native chicken feathers (10 g) in 500 ml of dimethyl sulfoxide were heated in a reflux condenser at 100 °C for 2 h. Soluble keratin was then precipitated by addition of cold acetone (1 L) at −70 °C for 2 h, followed by centrifugation at 10000×g for 10 min. The resulting precipitate was washed twice with distilled water and dried at 40 °C in a vacuum dryer. One gram of quantified precipitate was dissolved in 20 ml of 0.05 mol/L NaOH. The pH was adjusted to 8.0 with 0.1 mol/L Tris and 0.1 mol/L HCl and the solution was diluted to 200 ml with 0.05 mol/L Tris-HCl buffer (pH 8.0).

Keratinolytic activity determination

The keratinolytic activity was assayed as follows: 1.0 ml of crude enzyme properly diluted in Tris-HCl buffer (0.05 mol/L, pH 8.0) was incubated with 1 ml keratin solution at 50 °C in a water bath for 10 min, and the reaction was stopped by adding 2.0 ml 0.4 mol/L trichloroacetic acid (TCA). After centrifugation at 1450×g for 30 min, the absorbance of the supernatant was determined at 280 nm (UV-2102, UNICO Shanghai Corp., China) against a control. The control was prepared by incubating the enzyme solution with 2.0 ml TCA without the addition of keratin solution.

One unit (U/ml) of keratinolytic activity was defined as an increase of corrected absorbance of 280 nm (A_{280}) (Gradišar *et al*., 2005) with the control for 0.01 per minute under the conditions described above and calculated by the following equation:

$$U=4\times n\times A_{280}/(0.01\times 10), \quad (1)$$

where n is the dilution rate; 4 is the final reaction volume (ml); 10 is the incubation time (min).

Electron microscopy

To characterize the degradation of keratin-containing substrates, culture broths containing feathers, hair and silk were filtered and washed twice by distilled water. The substrates were then dried with a Hitachi HCP-2 critical point dryer and plated with Eiko IB-5 ion coater. The specimens were then observed with XL30-ESEM environment scanning electron microscopy.

Amino acids analysis

Amino acids analysis was performed on an amino acid analyzer L-8800 (Hitachi) after hydrolysis of the sample of cell free culture in 6 mol/L HCl for 24 h at 110 °C.

Determination of residual hydrolysates

The residual hydrolysates were composed of cells and undigested feathers. After cultivation, cultures containing the residual hydrolysates were centrifuged (1450×g), and filtered through Xinhua filter paper, and then dried to a constant weight (W_2, sum of the residual hydrolysates and filter paper). The weight of the residual hydrolysates was determined by sub-

tracting the weight of the filter paper (W_1, dried to a constant weight) from W_2.

RESULTS AND DISCUSSION

Identification and mutagenesis of strain KD-1

Light and electron micrographies showed that KD-1 is a single rod-shaped, Gram-positive bacterium capable of endospore formation in the mid-log phase. Carbohydrate metabolism results showed 97.5% similarity of the isolate to *B. subtilis*. The 16S rDNA sequence showed high levels of sequence similarity to the species *B. subtilis* (99%) (Fig.1). Phylogenetic analysis based on 16S rDNA sequences showed that the isolate is closely related to *B. subtilis* strains of KCC103 and MG-1 with the sequences accession Nos. AY973493.1 and DQ408585.1, respectively.

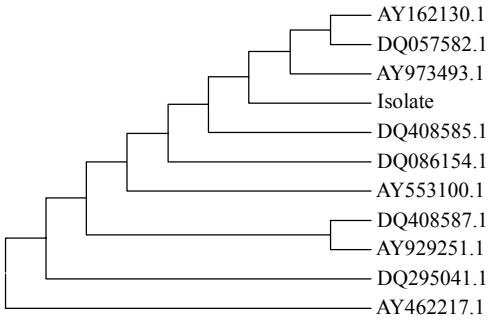

Fig.1 Phylogenetic tree based on 16S rDNA sequence of the isolate KD-1 and selected *Bacillus subtilis* strains from the database
The sequences were aligned using ClustalX program and the phylogenetic tree was booted by MEGA3 software

MNNG mutagenesis resulted in the isolation of two mutants from a casein plate, designated as KD-N1 and KD-N2, respectively (Fig.2). Keratinolytic activity assay demonstrated that KD-N2 ((60.9±0.87) U/ml) was about 2.5 times that of the wild-type strain ((24.3±1.31) U/ml) (Fig.3). The residual hydrolysates of KD-N2 were less than those of the wild-type strain (Fig.3).

Previous literatures have documented the isolation of keratinase-producing strains from *B. subtilis*, *B. licheniformis*, *B. pumilis*, *B. cereus*, *B. halodurans* and *B. pseudofirmis* (Williams *et al*., 1990; Takami *et al*., 1999; Rozs *et al*., 2001; Kim *et al*., 2001; Gessesse *et al*., 2003; El-Refai *et al*., 2005) and fermentation studies concerning their respective keratinase production. However, application of chemical mutagenesis to improve keratinase production has not yet been reported. Our results from this study demonstrated the feasibility of using MNNG to generate desirable mutants and screen them. The mutant strain KD-N2 produced higher keratinolytic activity than the wild-type strain, and the former degraded feathers intensively.

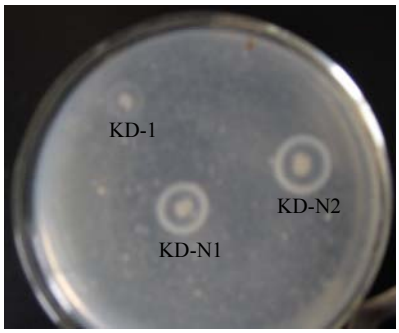

Fig.2 Production of clearing zones in casein agar by the wild-type strain (KD-1) and two mutants (KD-N1 and KD-N2)
The strains were inoculated with stick and plates incubated at 37 °C for 48 h

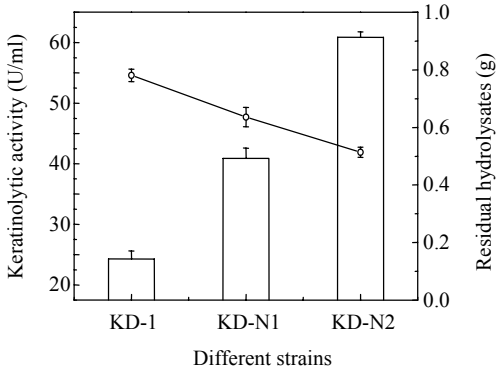

Fig.3 Keratinolytic activity (U/ml) and residual hydrolysates of the wild-type strain (KD-1) and two mutants (KD-N1 and KD-N2)
Cultivations were performed with 5% inoculum at 37 °C and 200 r/min for 24 h. Column: Keratinolytic activity; Dot: Residual hydrolysates

Effects of substrates on keratinolytic activity

As like most keratinolytic microorganisms, strain KD-N2 produced inducible keratinase when keratin-containing materials such as feathers, silk, hair and wool were used as sole substrates. Feather was the optimal substrate for keratinase production

(Fig.4) and the medium containing a feather concentration of 10 g/L was better for keratinase production than that of other feather concentrations (Table 1). Among all the keratin-containing substrates, feather was mostly utilized, followed by hair and wool. Silk was firstly utilized as substrate for keratinase production. Both α-keratin (from hair and wool) and β-keratin (from feathers and silk) can be utilized as substrates. Electron micrography studies showed the degradation of feathers, silk and hair during cultivation process (Fig.5). Soluble proteins of casein, skim milk powder and peptone failed to induce keratinase production. The ability of *B. subtilis* KD-N2 to use different keratin-containing substrates makes it applicable to both keratin-degradation and keratinase production.

Table 1 Effects of feather content on keratinase production and residual hydrolysates

Feather content (g/L)	Keratinolytic activity (U/ml)	Residual hydrolysates (g)
1	36.2±0.53	0.011±0.003
5	55.4±3.22	0.152±0.007
10	70.4±2.62	0.398±0.010
15	67.8±1.51	0.656±0.01
20	57.6±2.82	1.012±0.011

The cultivations were performed at initial pH 7.0, 28 °C and 200 r/min for 24 h with 5% 16-h-old inoculum

Effects of cultural conditions on keratinase production

The effects of initial pH, cultivation temperature, age of inoculum and inoculum size on keratinase production and residual hydrolysates were further investigated in terms of feather substrates.

The initial optimal pH for keratinase production was 7.5 and the medium pH increased to a relative state level of about 8.5 during cultivation (data not shown); the increased pH was caused by the production of ammonia and alkaline compounds (Table 2). The residual hydrolysates decreased gradually with the initial medium alkalinity.

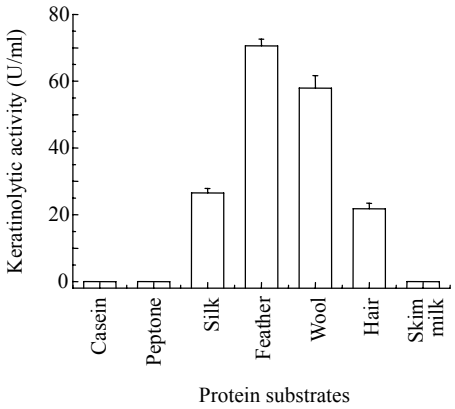

Fig.4 Keratinase production on different substrates
Cultivation was maintained at 37 °C and 200 r/min with 5% of 20-h-old inoculum for 24 h

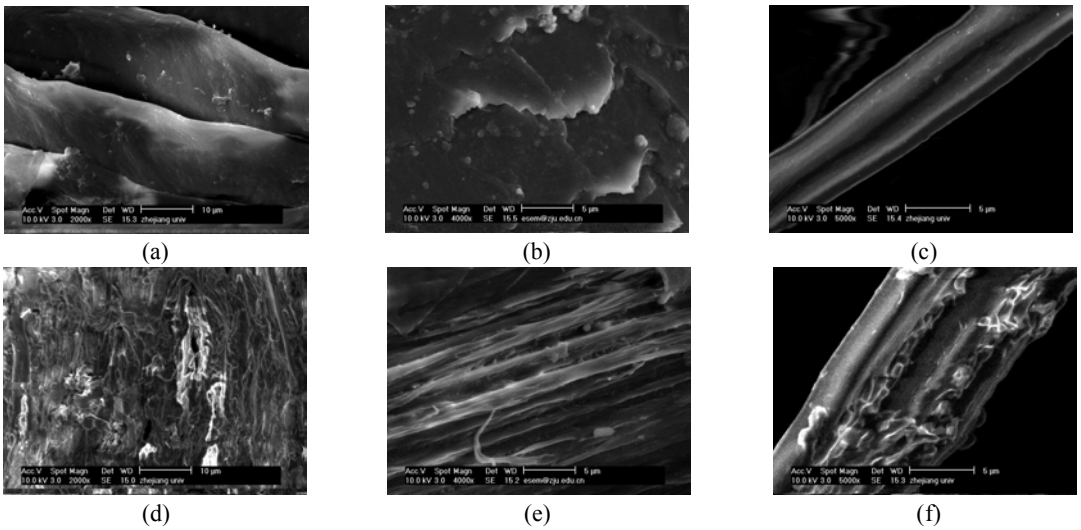

Fig.5 Degradation of keratin-containing substrates by KD-N2 in submerged cultivation. (a) Feathers control; (b) Hair control; (c) Silk control; (d) Feathers 24 h; (e) Hair 72 h; (f) Silk 72 h
Substrates of feathers, hair and silk in culture broths were filtered, washed with distilled water, dried with Hitachi HCP-2 critical point dryer, and then plated with Eiko IB-5 ion coater. The specimens were examined with XL30-ESEM environment scanning electron microscopy

Table 2 Effects of initial pH on keratinase production and residual hydrolysates

Initial pH	Keratinolytic activity (U/ml)	Residual hydrolysates (g)
6.5	63.6±1.83	0.765±0.012
7.0	71.6±6.43	0.665±0.012
7.5	82.2±5.93	0.633±0.014
8.0	81.8±5.83	0.549±0.015
8.5	72.0±3.80	0.548±0.013

The cultivations were performed at 28 °C and 200 r/min for 24 h with 5% 16-h-old inoculum in 10 g/L feather substrate

The optimal temperature for keratinase production was 23 °C (Table 3). As the cultivation temperature increased from 23 to 42 °C, the keratinase produced decreased rapidly. *Bacillus* sp. usually showed optimal keratinase production at temperatures ranging from 30 to 50 °C, for example, *Bacillus* sp. FK 46 at 37 °C (Suntornsuk and Suntornsuk, 2003), *B. licheniformis* PWD-1 at 50 °C (Williams *et al*., 1990).

Table 3 Effects of cultivation temperature on keratinase production and residual hydrolysates

Temperature (°C)	Keratinolytic activity (U/ml)	Residual hydrolysates (g)
18	51.4±3.86	0.573±0.013
23	83.6±2.11	0.605±0.017
28	70.6±2.23	0.538±0.013
32	43.6±3.17	0.473±0.036
37	42.4±5.50	0.499±0.046
42	21.8±3.54	0.766±0.019

The cultivations were performed at initial pH 7.0 and 200 r/min for 24 h with 5% 16-h-old inoculum in 10 g/L feather substrate

Inoculum size is very important factor affecting cell growth and product formation. The inoculum size of 2% (v/v) was optimal for keratinase production, followed by the inoculum size of 5% (v/v). The keratinase produced decreased with the increase of inoculum size, but the amounts of residual hydrolysates increased (Table 4). However, there is no feasible explanation for this experimental phenomenon.

Table 4 Effects of inoculum size on keratinase production and residual hydrolysates

Inoculum size (%)	Keratinolytic activity (U/ml)	Residual hydrolysates (g)
2	71.4±4.13	0.254±0.014
5	70.0±2.23	0.367±0.010
8	57.8±1.91	0.395±0.004
10	56.8±0.60	0.404±0.010

The cultivations were performed at initial pH 7.0, 28 °C and 200 r/min for 24 h with 16-h-old inoculum in 10 g/L feather substrate

The age of inoculum slightly affected keratinase production, and 16 h was optimal for keratinase production. The residual hydrolysates also varied slightly except for 8-h-old inoculum (Table 5). They were composed of undigested feathers and bacterial cells; cultivation conditions affected keratinase production and cell growth, thus the amounts of residual hydrolysates varied under different cultivation parameters. It was found that the optimal conditions were as follows: initial pH 7.5, inoculum size of 2%, age of inoculum 16 h and temperature 23 °C. The cultivation process was investigated and analysed. During submerged cultivation the maximum keratinolytic activity was achieved at about 30 h, then it started to decrease (Fig.6).

Table 5 Effects of age of inoculum on keratinase production and residual hydrolysates

Age of inoculum (h)	Keratinolytic activity (U/ml)	Residual hydrolysates (g)
8	68.6±2.11	0.551±0.009
12	70.6±3.12	0.515±0.008
16	73.2±4.39	0.499±0.017
20	68.0±4.80	0.483±0.014
24	67.4±5.19	0.519±0.014

The cultivations were performed at initial pH 7.0, 28 °C and 200 r/min for 24 h with 5% inoculum in 10 g/L feather substrate

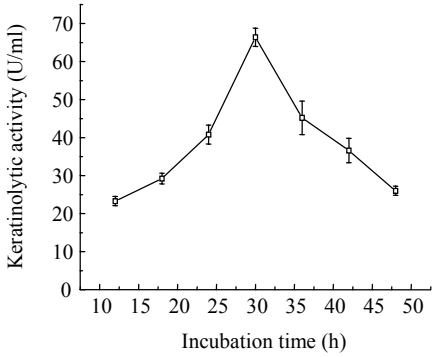

Fig.6 Time course production of keratinase using 1 g feathers as substrate

The cultivation was incubated with 2% of 16-h-old inoculum at 28 °C and 200 r/min

Amino acids production

The mutant strain KD-N2 degraded feathers and produced amino acids in submerged cultivation. Essential amino acids, threonine, valine, methionine, isoleucine, phenylalanine and lysine, were all produced in the culture, and the most abundant amino acid produced was cysteine, reaching 0.1540 mg/ml

(Table 6), which may be due to the high disulfide content of feather keratin. Based on the above result, the degraded feathers and fermented broth containing bacterial cells and amino acids can be used as feed additives or fertilizers.

Table 6 Amino acids and ammonia production by KD-N2 after cultivation for 30 h with 5% of 16-h-old inoculum at 28 °C and 200 r/min

Amino acids	Content (mg/ml)	Amino acids	Content (mg/ml)
Serine	0.0369	Leucine	0.0369
Threonine	0.0270	Tyrosine	0.0508
Glycine	0.0382	Phenylalanine	0.0136
Glutamic acid	0.0640	Lysine	0.0236
Alanine	0.0238	Arginine	0.0208
Cysteine	0.1540	Histidine	0
Valine	0.0627	Proline	0
Methionine	0.0136	NH_3	0.4424
Isoleucine	0.0188		

The precise mechanism underlying keratinolysis has yet to be elucidated. It has been proposed that the first step in keratin degradation involves deamination, which creates an alkaline environment needed for substrates swelling, sulphitolysis, and proteolytic attack (Kunert, 2000). In the case of KD-N2, NH_3 was produced when feathers were used as sole substrate (Table 6), and electron micrographies clearly showed the degradation of feathers, hair and silk.

CONCLUSION

In this paper we report the successful application of MNNG as a mutagenesis tool to generate, from a wild-type keratinase-producing *B. subtilis* strain, mutants with elevated keratinolytic activity and their convenient screening on casein agar plates. To our best knowledge, this is the first report of using chemical mutagenesis to improve keratinolytic activity of keratinase-producing microbes. The isolated mutant KD-N2, capable of degrading several keratin-containing materials including feathers, hair, silk and wool, was shown to produce inducible keratinolytic activity at levels 2.5 times that of the wild-type strain in feather substrates. Silk was firstly utilized as substrate for keratinase production by *Bacillus* sp. The maximum keratinolytic activity produced by KD-N2 mutant in feather fermentation was achieved at 30 h. The newly isolated mutant KD-N2 shows remarkable feather-degrading capabilities and thus may find potential applications in keratinase production and feather waste utilization.

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