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Full Length Research Paper

Characterization of a new feather-degrading bacterium from *Calotes versicolor* feces

Xiao-Yu Yue[1,2*#], Bin Zhang[2#], Dan-Dan Jiang[3], Yan-Juan Liu[1] and Tian-Gui Niu[2]

[1]Department of Quality Detection and Management, Henan University of Animal Husbandry and Economy, Henan 450011, China.
[2]College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, China.
[3]Beijing Homeport Food ingredients Trading Company, Beijing, China.

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A total of 842 spore-forming strains were isolated from 221 animal feces samples, in which a new feather-degrading bacterium identified as *Bacillus* sp. 50-3 based on morphological, biochemical and 16S rDNA tests was isolated from *Calotes versicolor* (an agamid lizard) feces. The bacterium can degrade native feather completely at mild conditions and in much shorter time (36 h) when using chicken feather as the sole carbon and nitrogen source. It presented optimum growth and maximum keratinase activity (680±25 U/ml, 36 h) at 37°C and pH 7.0 in feather meal medium. The keratinase of *Bacillus* sp. 50-3 was active on feather keratin as substrate and presented optimum additive quantity of 1.0% (w/v). So the high efficiency of *Bacillus* sp. 50-3 on feather-degradation suggested its potential use in biotechnological processes, especially in decreasing the environment pollution.

Key words: Animal feces, *Bacillus,* chicken feathers, keratinase, optimum conditions.

INTRODUCTION

The micro-organisms and their enzymes play an important role in improving the industrial process. Because the enzyme-catalyzed reactions are highly efficient, easily selective and less pollution, usually they require mild conditions and less energy which lead to the lowering of costs (Cherry and Fidantsef, 2003). And the world market has a big need for industrial enzymes which is estimated to be about 1.6 billion $US, split among food enzymes (29%), feed enzymes (15%) and general technical enzymes (56%) (Outtrup and Jorgensen, 2002). Thus, study on isolating new enzymes and new enzyme-producing strains used in the industrial conversions are significant.

Bacillus species are attractive industrial organisms because of their high growth rates leading to short fermentation cycle times; their capacity to secrete proteins into the extracellular medium and the GRAS (generally regarded as safe) status by the food and drug administration for species, such as *Bacillus subtilis* and *Bacillus licheniformis* (Marcus et al., 2004).

Keratin is the most abundant structural protein in skin, horn, hair, wool and feathers, which is rich in α-helix or β-sheet linked with cystine bridges (Bockle et al., 1995). So the commonly known proteases could not degrade the keratin to a large degree; however, the keratinase purified from different microorganisms and characterized to date all act as proteases have a high level of activity on it. In recent years, there have been many reported microorganisms to produce the enzyme such as *Bacillus* species (Bockle et al., 2005; Kim et al., 2001; Lin et al., 1992; Williams et al., 1990), fungi (Bockle et al., 1995; Chao et al., 2007; Gradisar et al., 2000; Santos et al., 1996) and others (Bernal et al., 2006; Nam et al., 2002; Riessen and Antranikian, 2001; Riffel et al., 2003; Sangali and Brandelli, 2000; Thys et al., 2004). However, there is still

*Corresponding author. E-mail: yuerain@163.com. Tel: +86-371-65751570. Fax: +86-371-65765762.

#These authors contributed equally to this work.

a need to find the new strains and enzymes that can be applied in the biotechnological processes involving keratin-containing wastes from poultry and leather industries, especially in the development of non-polluting processes (Onifade et al., 1998; Shih, 1993; Wang et al., 2007).

In this study, we isolated 842 strains of spore-forming bacteria from 221 animal feces samples obtained from Beijing Zoo, China and one strain identified as *Bacillus* sp. 50-3 was found to have high keratinase activity. Some features, such as growth conditions, keratinase producing conditions were also determined.

MATERIALS AND METHODS

Chemicals

Chemicals used in the bacterial cell cultivation and taxonomic studies were from Oxoid Ltd (Basingstoke, UK) and Merck AG (Darmstadt, Germany); Azokeratin was synthesized based on the method described elsewhere (Riffel et al., 2003); Chicken feather was from Beijing Huadu chicken factory (Beijing, China).

Sample collection

Samples were collected from Beijing Zoo, China. These fresh samples were from 221 animals such as Snow leopard, Siberian Tiger, Sika Deer, Kangaroo, Racoon, Squirrel Monkey, Takin, Addax, Giant Panda, Lizard, Cougar and Cobra, which were all fed in Beijing Zoo. These samples in sterile 100 ml glass bottles were carried back to laboratory.

Strain isolation

Microorganisms were extracted by suspending 1 g of each feces sample in 9 ml sterile 0.9% (w/v) saline solution using the end-point dilution technique. Firstly, the test tube with feces concentration of 0.1 g/ml was treated for 10 min at 80°C to isolate only spore-forming bacteria. 0.1 ml of each dilution from the proper concentration was spread on agar plates containing the feather meal and the resulting plates were incubated for 72 h at 37°C. The feather-meal agar plates contained: [10 g feather meal, 0.5 g NaCl, 0.3 g K_2HPO_4, 0.4 g kH_2PO_4, 15 g agar, per litre; pH 7.0 to 7.2].

The colonies with clear zones formed by hydrolysis of feather keratin were evaluated as keratinase producers. The strains whose zone diameter is 5-fold longer than the colony diameter were selected and used in all further investigations.

Growth determination

The inoculum culture is the Luria-Bertani (LB) [10 g bactotryptone, 5 g yeast extract, 10 g NaCl, per litre; pH 7.2-7.6]; 20 ml of the feather meal medium [10 g feather meal, 0.5 g NaCl, 0.3 g K_2HPO_4, 0.4 g kH_2PO_4, per litre; pH 7.0 to 7.2] in 100 ml Erlenmeyer flasks were inoculated from a 10^6 colony forming units (CFU)/ml culture and then cultivated by shaking at 150 rpm for 72 h. Growth of the isolation was tested within the temperature range of 25 to 70°C (25, 37, 45 and 70°C) and pH range of 4.0 to 11.0 (4.0, 5.0, 7.0, 8.0 and 11.0). Bacterial growth was monitored by measuring the CFU/ml, as described elsewhere (Sangali and Brandelli, 2000).

Taxonomic studies

The morphological and physiological characteristics of the isolated bacteria were compared with data from Bergey's manual of determinative bacteriology (Holt et al. 1994).

The strain DNA was extracted from cells after cultivation for 24 using the TIANamp bacteria DNA kit (Beijing TIANGen Biotech, China) according to the manufacturer's instructions. The 16S rDNA gene was amplified by PCR using primers 27f (5´-GAGAGTTTGATCCTGGCTCAG-3´), 530f (5´-GTGCCAGCAGC CGCGG-3´) and 1541r (5´-AAGGAGGTGATCCAGCCGCA-3´), corresponding to *Escherichia coli* 16S rRNA gene position. The PCR products were purified and sequenced by the Beijing HuaDa Gene Study Center, China with the ABI 3730 automated sequencer. The 1,477 bp sequence was submitted to Genbank (accession number EU365432) and was searched for its homologous sequences by BLAST algorithm method. The 16S rDNA sequences were reversed, aligned and compared with similar database sequences using the Clustal X program, version 1.83 (Thompson et al., 1997). A phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) employing the program molecular evolutionary genetic analysis (MEGA) 3.1 (Kumar et al., 2004). The branching pattern was checked by 1,000 bootstrap replicates.

Enzyme production

The organism was cultivated for 36 h in feather meal medium, from a 10^6 CFU/ml culture. Samples were centrifuged at 10,000×*g* for 10 min and the supernatant fluid used as a crude enzyme preparation. To study cell-associated enzyme, the collected cells were washed twice with 50 mM Tris buffer (pH 8.0) and suspended in the same buffer. The cells were disrupted by sonication for 10 min at 4°C and centrifuged at 10,000×*g* for 10 min. The supernatant fluid was used to determine the enzyme activity.

Enzyme assays

Keratinase activity was assayed with azokeratin as a substrate according to the modified method described by Riffel et al. (2003). The reaction mixture contained 200 µl of enzyme preparation and 1.6 ml of 10 g/l azokeratin in 50 mM glycine/NaOH buffer, pH 10.0. The mixture was incubated for 15 min at 60°C. The reaction was stopped by adding the trichloroacetic acid to a final concentration of 100 g/l. After centrifugation at 10,000×*g* for 10 min, the absorbance of the supernatant fluid was determined at 440 nm. One unit of enzyme activity was the amount of enzyme that caused a change of absorbance of 0.01 at 440 nm for 15 min at 60°C.

Effect of the different substrates on the keratinase activity

Three different substrates were tested in the experiment. The three media contain [10 g of human hair, native chicken feather and the cattle tendon respectively, 0.5 g NaCl, 0.3 g K_2HPO_4, 0.4 g kH_2PO_4, per litre; pH 7.0-7.2], 20 ml of each in 100 ml Erlenmeyer flasks was inoculated from a 10^6 CFU/ml culture and cultivated by shaking at 150 rpm for 72 h. The keratinase activity was tested every 12 h.

RESULTS

Isolation of the spore-forming bacteria and selection of the keratin-degrading strain

A total of 221 feces samples from different animals were collected to isolate the spore-forming bacteria. All of 842 pure cultures of spore-forming bacteria were obtained and 196 strains observed the clear zone on feather-meal agar plates. Among them, strain 50-3 was isolated from

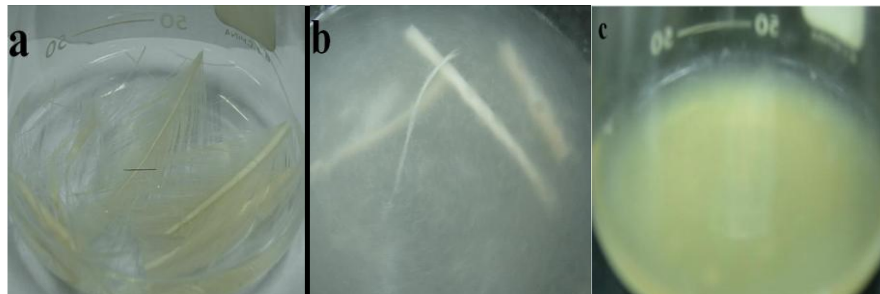

Figure. 1 The native Chicken feather was degraded after 0 h (a), 24 h (b) and 36 h (c), respectively at 37°C by strain 50-3.

C*alotes versicolor* (an agamid lizard) feces. The degrading zone diameter of it is about 6-fold longer than the colony diameter. Also, it could degrade the chicken feather absolutely, after 36 h culturing on the native chicken feather as the sole carbon, nitrogen and energy source (Figure 1). So the strain 50-3 was chosen for further characterization.

Identification of strain 50-3

The identification of strain 50-3 was based on the morphological, the physiological characteristics and the 16S rDNA tests. Some characters were summarized in Table 1. Together with morphological and physiological characteristics assigned the strain 50-3 to the genus *Bacillus*. The closest species were *Bacillus velezensis* (Cristina, 2005) and *Bacillus vallismortis* (Roberts et al., 1996), which differed from 50-3 in the Tween-80 hydro-lysis and producing arginine dihydrolase, respectively.

The phylogenetic tree based on the comparison of 16S rRNA sequences of reference strains was constructed (Figure 2). The isolate 50-3 formed a highly compact phylogenetic cluster with the following species: *B. velezensis*, *B. vallismortis*, *B. subtilis* and *B. amyloliquefaciens*.

Optimal growth conditions

The strain *Bacillus* sp. 50-3 could grow at 25 to 70°C and pH 4.0 to 11.0, with an optimum at 37°C and pH 7.0. It grew slowly at 25 and 70°C, with less cell number compared with 37 and 50°C. It grew at approximately the same rate at pH 5.0 and 7.0, but more slowly at pH 4.0 and 11.0 (data not shown). Interestingly, the all final pH became around 8.0 after being cultivated for 72 h.

Production of keratinase

During cultivation of *Bacillus* sp. 50-3 in feather meal medium at 37°C, less activity of keratinase was found in the cell lysate and the highest amount of keratinase activity was produced in the supernatant fluid of the culture medium (data not shown). At the optimal growth conditions, the keratinase reached a maximum activity at 36 h, coinciding with the end of exponential phase, then decreased and increased again at 72 h (Figure 3).

The effects of temperature and pH on the production of keratinase were investigated. Maximum enzyme activity was observed during cultivation at 37°C (680±25 U/ml at 36 h), followed by 25°C (465±18 U/ml at 48 h), but less enzyme activity at 50 and 70°C (165 ±36 U/ml at 36 h and 116± 22 U/ml at 24 h, respectively). At 37°C, with the different initial pH, the maximum keratinase activity appeared at different time, especially at pH 4.0, the enzyme activity was higher with increasing cultivation time before 60 h (data not shown) and furthermore, the most proper pH was 7.0. Thus, the maximum keratinase activity was observed at 37°C with initial pH 7.0 and cultivation time 36 h in feather meal medium.

Effect of different substrates on keratinase activity

Three of the different substrates were used to test the effect on keratinase activity (Figure 4). The chicken feather substrate presented greatly increases the keratinase production. On the other hand, the human hair and cattle tendon suggested relatively low enzyme activities.

Effect of different feather meal concentration

The effect of different feather meal concentrations on keratinase activity was investigated. It was seen that the feather meal concentration on the keratinase activity was not important (Figure 5). The optimum concentration was 1.0% (w/v) as the keratinase activity was relatively high.

DISCUSSION

In earlier reports, the spore-forming strains have been isolated from variously ecosystems such as soil, water and food (Lin et al., 1999; Shih et al., 2005). The isolates from animal feces were less reported (Nakada and Ohta,

Table 1. Morphological and physiological characteristics of the strain 50-3.

Morphological characteristic		Physiological characteristic	
Form	Rods	Catalase	Positive
Size	0.5×1-2 μm	Oxidase	Positive
Gram stain	Positive	Nitrate reduction	Positive
Spore	Positive	Voges-Proskauer test	Positive
Acid from		Citrate	Negative
D-xylose	Positive	Propionate	Positive
Galactose	Negative	alginate	Negative
D-fructose	Positive	Gelatin liquefaction	Positive
Mannose	Positive	Starch hydrolysis	Positive
Mannitol	Positive	Casein hydrolysis	Positive
Cellobiose	Positive	Tween 80 hydrolysis	Positive
Maltose	Positive	Lysine decarboxylase	Positive
Melitose	Negative	Arginine dihydrolase	Positive
D-trehalose	Positive	Urease	Positive
Saccharose	Positive	DNAse	Positive
		Indol production	Positive

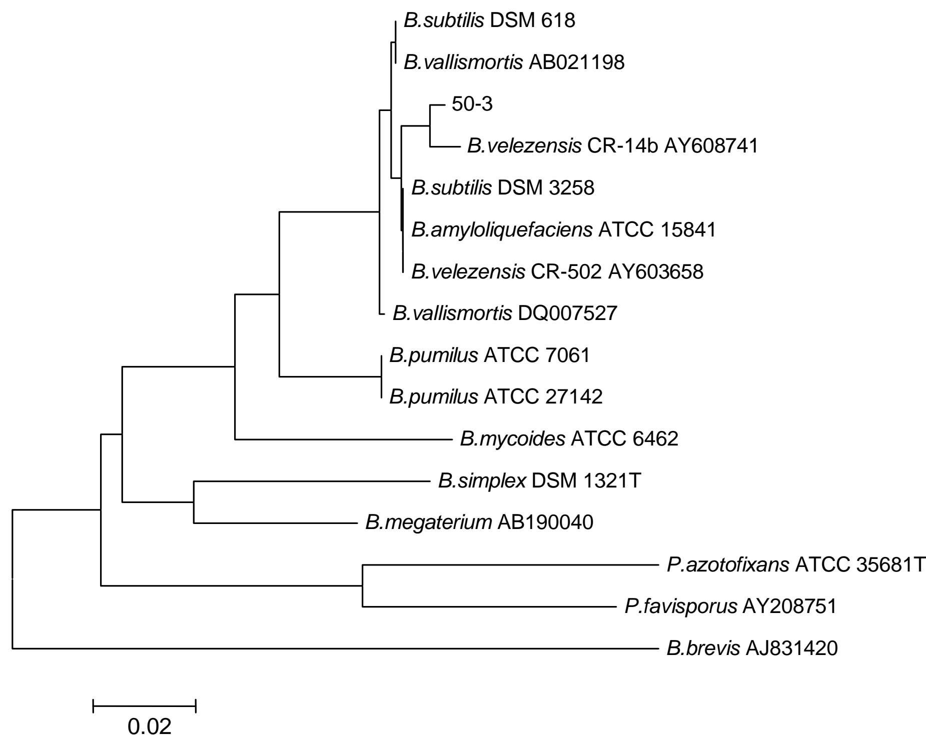

Figure. 2 Strain 50-3 and selected bacteria comparisons of phylogenetic tree based on 16S rDNA sequence. The branching pattern was generated by the neighbor-joining method. The bar indicates the 2% estimated difference in nucleotide sequences.

1999; Swiecicka et al., 2002). In this study, there are so many spore-forming bacteria in animal feces, which is an feces, which could be as a microorganism of environmental origin. As it is known that C. *versicolor* is an agamid lizard found widely in Asia, the main food of which is organic matter-rich environment. The most active strain 50-3 was isolated from *C. versicolor* (an agamid lizard) insects such as crickets, beetles and spiders. Therefore, there could be some microorganisms colonizing its gastrointestinal tract to degrade the insects' scute, which is

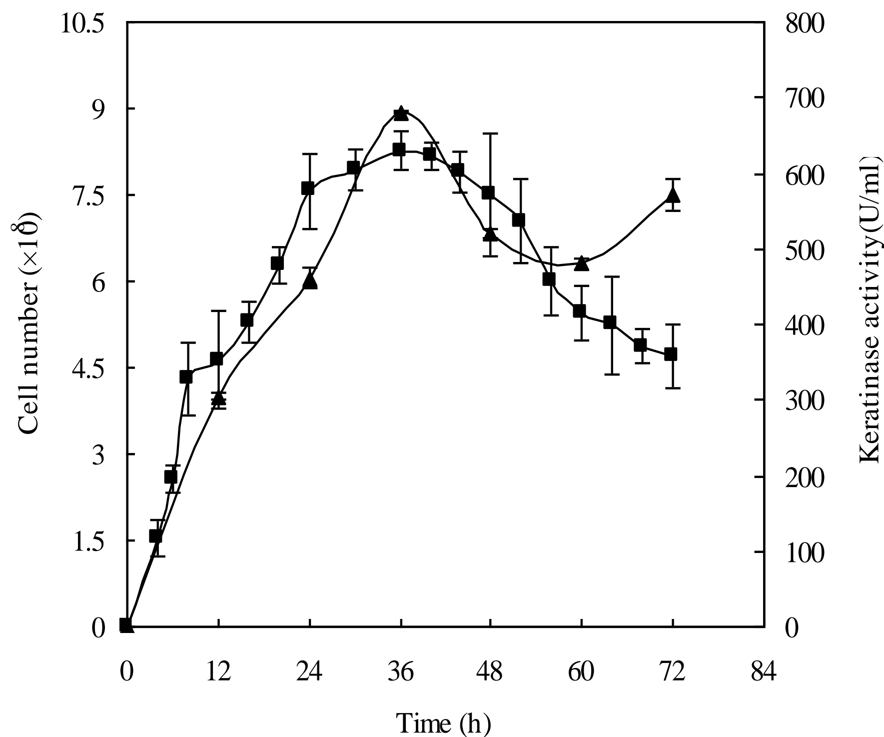

Figure. 3 Time course of the growth and keratinase production of *Bacillus* sp. 50-3 grown on 1.0% feather-meal medium at 37°C by shaking at 150 rpm. Closed diamond: Cell, closed triangle: Keratinase activity. Each point represents the mean±SEM of three independent experiments.

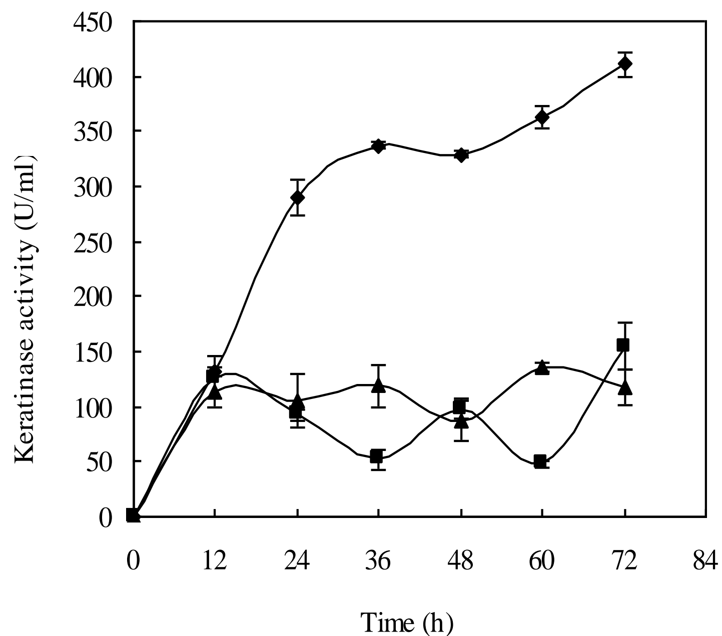

Figure. 4 Effect of three different substrates on keratinase activity from *Bacillus* sp. 50-3. To measure activity, the enzyme was assayed at each 12 for 72 h by the method in above. (closed diamond) native chicken feather; (closed triangle) cattle tendon; (closed square) human hair. Each point represents the mean±SEM of the three independent experiments.

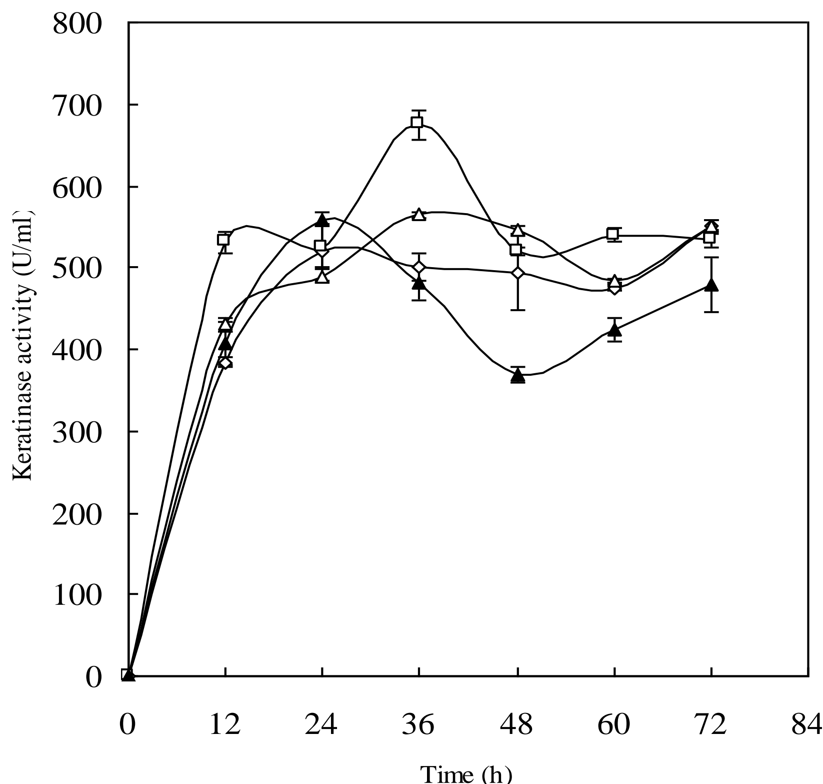

Figure. 5 Effect of different feather concentration on the keratinase activity from *Bacillus* sp. 50-3. To measure activity, the enzyme was assayed at each 12 for 72 h by the method in above. Open diamond 0.5%, open square 1.0%, open triangle 1.5% and closed triangle 2.0%. Each point represents the mean±SEM of three independent experiments.

full of keratin and facilitate efficient digestion (Angelis et al., 2006; Bevilacqua et al., 2003).

The morphological and physiological characteristics and the 16S rRNA sequence indicated that strain 50-3 was within the *B. subtilis* group. In a previous paper on taxo-nomy, species included in the *B. subtilis* group are the following: *B. velezensis*, *Bacillus atrophaeus*, *B. mojavensis*, *Bacillus malacitensis*, *Bacillus axarquiensis*, *Bacillus nematocida*, *Bacillus vallismortis*, *B. subtilis* and *Bacillus amyloliquefaciens* (Roberts et al., 1994, 1996; Wang et al., 2007). The 16S rDNA sequence showed high simi-larity (>99%) to some strains of *B. velezensis*, *B. vallismortis* and *B. subtilis*, a level of similarity which was greater than the borderline 98.7% for defining bacterial genomic spe-cies (Stackebrandt and Ebers, 2006; Stackebrandt and Goebel, 1994). Thus, only the 16S rDNA sequencing was not helpful in determining which species the strain 50-3 belonged to and its accurate identification needed more study. Therefore, the strain was named *Bacillus* sp. 50-3 at present.

Bacillus species are an important source of industrial enzymes which make up about 50% of the total enzyme market (Marcus et al., 2004). But the newly isolated *Bacillus* sp. 50-3 is a novel *Bacillus* strain to degrade feather keratin with high keratinase activity. Compared with most other feather degrading strains (El-Refai et al., 2005; Lin et al., 1999; Nam et al., 2002; Riffel et al., 2003), it could degrade the native feather in much shorter time (36 h) and have a relatively high keratinase activity (680±25 U/ml) at the same conditions.

The strain *Bacillus* sp. 50-3 could grow at wide tempe-rature and pH conditions. During cultivation both at initial low pH 4.0 and high pH 11.0 in feather meal medium, pH would come to around 8.0 which indicated the keratin hydrolysis (Sangali and Brandelli, 2000). At low initial pH, it presented the same result with the previous report (Riffel et al., 2003; Sangali and Brandelli, 2000). And at higher initial pH, the pH would decrease firstly, which might be caused by the keratin degradation and the free carboxyl increases in the medium to balance the pH which was proper for the strain growth.

The keratin is the abundant structural protein in feather and hair (Riessen and Antranikian, 2001) and the kerati-nase is inducible requiring keratin as an exogenous inducer (El-Refai et al., 2005). Therefore in feather medium, the keratianse could be largely inducible and its enzyme activity is higher than that in hair medium (Figure 4). The hair was difficulty to degrade; this might be caused by the hair keratin has much more α-helix structures which are difficulty to degrade than the feather keratin. Also in cattle

tendon, collagen is the main protein, so the keratinase activity was much lower even the cattle tendon was degraded absolutely. This was different from the result of the *Fervidobacterium islandicum* AW-1 (Nam et al., 2002).

The newly isolated strain 50-3 was a new *Bacillus* strain with high keratinase activity and it was from rather unorthodox sources, which indicated its novel characters. The strain could degrade the native feather in much shorter time at mild conditions. This suggested its potential use in biotechnological processes involving keratin hydrolysis to improve efficiency. In addition, the animal feces are rich in organic matter, which could lead to isolate much more useful microorganisms from them in the environment. Also, the keratianse character is under investigation, as far as the high biotechnological potential of the strain is concerned.

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