Characterization of a new bacterium with high alkaline keratinase activity from Calotes versicolor feces

Xiaoyu Yue[1, *], Bin Zhang[2], Jian Zou[1], Weifeng Chen[1], Na Yang[1]

[1]College of Food Engineering, Henan University of Animal Husbandry and Economy, Zhengzhou, Henan 450046, China. [2]Beijing Homeport Food Ingredients Trading Company, Beijing, China.

Received: March 20, 2017; accepted: July 3, 2017.

A total of 842 spore-forming bacterial strains were isolated from 221 animal feces samples, in which a new feather-degrading bacterium identified as *Bacillus sp*. 50-3 was isolated from Calotes versicolor (an agamid lizard) feces based on morphological, biochemical, and 16S rDNA tests. The bacterium degraded native feathers completely at mild conditions and in much shorter time (36 h) when chicken feathers were applied as the sole carbon and nitrogen source. The maximum keratinase activity and the optimum reaction time were 680 ± 25 U/ml and 36 h respectively at 37°C and pH 7.0 in feather meal medium. The optimal keratinase concentration of *Bacillus sp*. 50-3 with feather keratin as substrate was 1.0 % (w/v). The high efficiency of *Bacillus sp*. 50-3 in feather degradation suggests its potential applications in biotechnological processes, especially in decreasing environmental pollution.

Keywords: Animal feces; *Bacillus*; Chicken feathers; Keratinase; Optimum conditions.

[*]**Corresponding author:** Xiaoyu Yue, College of Food Engineering, Henan University of Animal Husbandry and Economy, No.6 Longzihu North Road, Zhengdong New District, Zhengzhou, Henan 450046, China. E-mail: yuerain@163.com.

Introduction

The microorganisms and their enzymes play an important role in improving the industrial process. The enzyme-catalyzed reaction, in addition to its advantages of highly efficient, easily selective, and less pollution, usually requires mild reaction conditions and less energy, which lead to the lowering of industrial costs [1, 2]. Therefore, any study on isolating new enzymes and new enzyme-producing bacterial strains used in the industrial conversions is significant.

Bacillus species, such as *Bacillus subtilis* and *Bacillus licheniformis* [3], are the attractive industrial microorganisms because of their high growth rates that lead to short fermentation cycle time, large capacity to secrete proteins into the extracellular medium, and the GRAS (generally regarded as safe) status defined by the Food and Drug Administration (FDA).

Keratins represent a group of filament-forming proteins that constitute the bulk of epidermal appendages such as hair, nails, claws, turtle scutes, horns, whale baleen, beaks, and feathers. Keratins can be classified as α- and β- types. Both categories show a characteristic filament-matrix structure [4]. The commonly known proteases could not degrade the keratin to a large degree. However, the keratinases that are purified from different microorganisms and are characterized as a protease have a high level of activity on keratin degradation. In recent years, there are many reports about different microorganisms to produce the keratinase enzyme such as *Bacillus* species [5-9], fungi [10-12], and others [13-19].

However, there is still needs to explore new microorganism strains and enzymes, especially the development of non-polluting processes, that can be applied in the poultry and leather industries involving keratin-containing wastes [2, 7, 19-22].

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

Material and methods

Chemicals

Chemicals used in the bacterial cell cultivation and taxonomic studies were obtained from Oxoid Ltd (Basingstoke, UK) and Merck AG (Darmstadt, Germany). Azokeratin was synthesized based on the method described by Riffel et al. [16]. Chicken feather was obtained from Beijing Huadu Chicken Factory (Beijing, China).

Fecal sample collection

Fecal samples were collected from Beijing Zoo (Beijing, China). These fresh samples were collected from 221 animals including 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. The collected fecal samples were stored in sterile 100 ml glass bottles and were carried back to laboratory for following experiments.

Strain isolation

Microorganisms were extracted by suspending 1 g of each fecal sample in 9 ml of sterile 0.9% (w/v) saline solution by using the end-point dilution technique. The test tube containing feces at the concentration of 0.1 g/ml was incubated at 80°C for 10 min to isolate spore-forming only bacteria. And then, 0.1 ml of each dilution was spread on feather meal agar plates (contains 10 g feather meal, 0.5 g NaCl, 0.3 g K_2HPO_4, 0.4 g kH_2PO_4, and 15 g agar in 1 liter media at the pH of 7.0-7.2). The plates were then incubated at 37°C for 72 h.

The colonies with clear zones formed by hydrolysis of feather keratin were identified as keratinase producers. The strain with its zone diameter 5-fold larger than that of the bacterial colony was selected for following experiments.

Growth determination

The selected bacteria strain was cultured in Luria-Bertani (LB) medium (contains 10 g bactotryptone, 5 g yeast extract, 10 g NaCl in 1 liter with the pH from 7.2 to 7.6). A concentration of 106 colony forming units (CFU)/ml culture was inoculated into 20 ml of the feather meal medium (contains 10 g feather meal, 0.5 g NaCl, 0.3 g K_2HPO_4, 0.4 g kH_2PO_4, in 1 liter soulution with the pH from 7.0 to 7.2) in 100 ml Erlenmeyer flasks, and then was cultivated at 37°C by shaking at 150 rpm for 72 h. Growth of the isolated bacterial strain was tested at the temperatures of 25°C, 37°C, 45°C, and 70°C; and at the different pH levels of 4.0 5.0, 7.0, 8.0, and 11.0. The growth of bacteria was evaluated by measuring the CFU/ml as described by Sangali and Brandelli [23].

Taxonomic studies

The morphological and physiological characteristics of the isolated bacteria were compared to the data from Bergey's Manual of Determinative Bacteriology [24].

The bacterial DNA was extracted from cells after cultivation for 24 h by using the TIANamp Bacteria DNA Kit (Beijing TIANGen Biotech, Beijing, China) according to the manufacturer's instructions. The 16S rDNA gene was amplified by using PCR technology with the primers of 27f (5′-GAGAGTTTGATCCTGGCTCAG-3′), 530f (5′-GTGCCAGCAGCCGCGG-3′), and 1541r (5′-AAGGAGGTGATCCAGCCGCA-3′), corresponding to *Escherichia coli* 16S rRNA gene. The PCR

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

Morphological characteristics		Physiological characteristics	
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

products were purified and sequenced by the Beijing HuaDa Gene Study Center (Beijing, China) with the ABI 3730 automated sequencer. The resulted 1,477 bp sequence was submitted to Genbank (accession number: EU365432) and was searched for its homologous sequences by using BLAST program. The 16S rDNA sequences were reversed, aligned, and compared to the similar sequences in database by using Clustal X program [25]. A phylogenetic tree was constructed thereafter by using the neighbor-joining method [26] with the software of Molecular Evolutionary Genetic Analysis (MEGA) 5.05 [27]. The branching pattern was checked by 1,000 bootstrap replicates.

Enzyme production

The microorganism cultivated for 36 h in feather meal medium was centrifuged at 10,000 g for 10 min. The supernatant was recovered as a crude enzyme preparation. To study cell-associated enzyme, the pelleted bacterial cells were washed twice by using 50 mM Tris buffer (pH 8.0) and re-suspended in the same buffer. The cells were disrupted by sonication at 4°C for 10 min, and then were centrifuged at 10,000 g for 10 min. The supernatant was collected to measure the enzyme activity.

Enzyme assays

Keratinase activity was measured through modified method described by Riffel *et al* [16] by using azokeratin as a substrate. 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 at 60°C for 15 min followed by adding trichloroacetic acid to a final concentration of 100 g/l to stop the enzymatic reaction. After centrifugation at 10,000 g for 10 min, the absorbance of the supernatant was measured at 440 nm wavelength. One unit of enzyme activity was defined as the amount of enzyme that caused a change of absorbance of 0.01 at 440 nm

wavelength under the reactive condition of 60°C for 15 min.

Effect of the different substrates on the keratinase activity

Three different substrates were tested in the experiments with the media (0.5 g NaCl, 0.3 g K_2HPO_4, 0.4 g kH_2PO_4, in 1 liter solution at the pH level from 7.0 to 7.2) containing 10 g of human hair, native chicken feather, and the cattle tendon, respectively. 20 ml of each substrate from a concentration of 106 CFU/ml bacterial culture was inoculated into a 100 ml Erlenmeyer flasks and cultivated by shaking at 150 rpm for 72 h. The keratinase activity was then tested every 12 h.

Results

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

A total of 221 feces samples from different animals were collected to isolate the spore-forming bacteria. Totally, 842 pure cultures of spore-forming bacteria were obtained and 196 strains were observed the clear zone on feather-meal agar plates with the strain 50-3 isolated from Calotes versicolor (an agamid lizard) feces. The diameter of degrading zone for strain 50-3 is about 6-fold longer than that of the bacterial colony. In addition, this strain could degrade the chicken feather completely after 36 h incubation with native chicken feather as the sole carbon, nitrogen, and energy source (Figure 1). Therefore, the strain 50-3 was selected for further characterization in this study.

Identification of strain of *Bacillus sp*.50-3

The identification of strain 50-3 was based on the morphological and physiological characteristics and the 16S rDNA tests. Some characters were summarized in Table 1. Together with morphological and physiological characteristics, the strain 50-3 was assigned to the genus *Bacillus*. The closest species were *B. velezensis* [28] and *B. vallismortis* [29], which differed from 50-3 in the Tween-80 hydrolysis and produced Arginine dihydrolase, respectively. The phylogenetic tree based on the comparison of 16S rRNA sequences of reference strains was constructed in Figure 2. The isolated strain 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°C to 70°C and pH 4.0 to 11.0 with an optimal growth condition at 37°C and pH 7.0. It grew slowly at 25°C and 70°C with less cell number than it at 37°C and 50°C. It grew at approximately the same rate at pH 5.0 and 7.0, but much slower at pH 4.0 and 11.0 (data not shown). Interestingly, all final culture pH levels were 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. The highest keratinase activity was found in the supernatant of the culture medium (data not shown). At the optimal growth conditions, the keratinase activity reached the maximum at 36 h, which coincided with the end of the cell exponential phase, and then, the enzymatic activity decreased. However, the enzymatic activity 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 at 37°C (680 ± 25 U/ml at 36 h) followed by 25°C (465 ± 18 U/ml at 48 h), while the less enzyme activity was observed at 50°C 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 remained at the high level when the cultivation time was increased before reaching 60 h (data not shown) with the proper pH at 7.0. Therefore, the optimal reaction conditions for feather meal

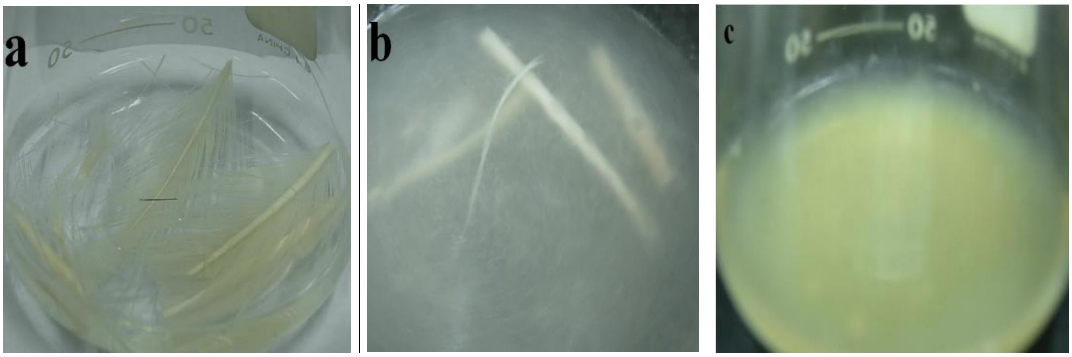

Figure 1. The native Chicken feather was degraded after 0 h (a), 24 h (b), and 36 h (c) respectively at 37°C by strain 50-3.

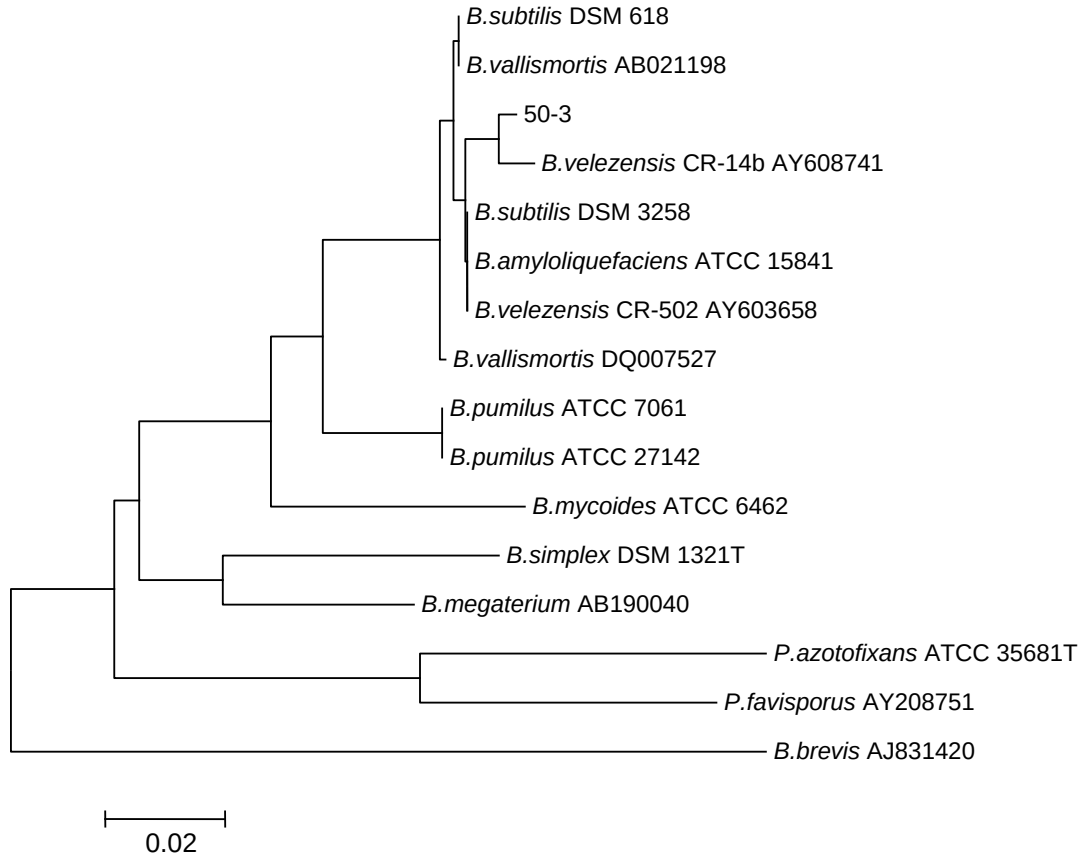

Figure 2. Strain 50-3 and selected bacteria comparisons of phylogenetic tree based on 16S rDNA sequence. The branching pattern was generated by the neighbor-joining method. The bar indicates the 2% estimated difference in nucleotide sequences.

medium experiments were set at 37°C with the initial pH at 7.0 and cultivation time 36 h.

Effect of different substrates on keratinase activity

Three different substrates were used to test the effect on keratinase activity (Figure 4). The chicken feather substrate test demonstrated the highest keratinase activity while the human hair and cattle tendon tests suggested relatively low enzyme activities.

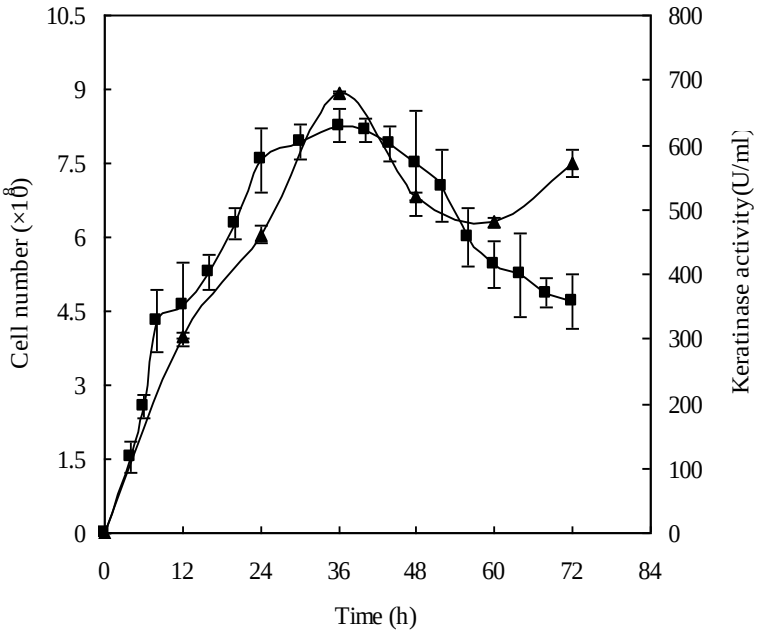

Figure 3. Time course of the growth and keratinase production of *Bacillus* sp. 50-3 grown on 1.0% feather-meal medium at 37°C by shaking at 150 rpm. Closed square: Cell, closed triangle: Keratinase activity. Each point represents the mean ± SEM of three independent experiments.

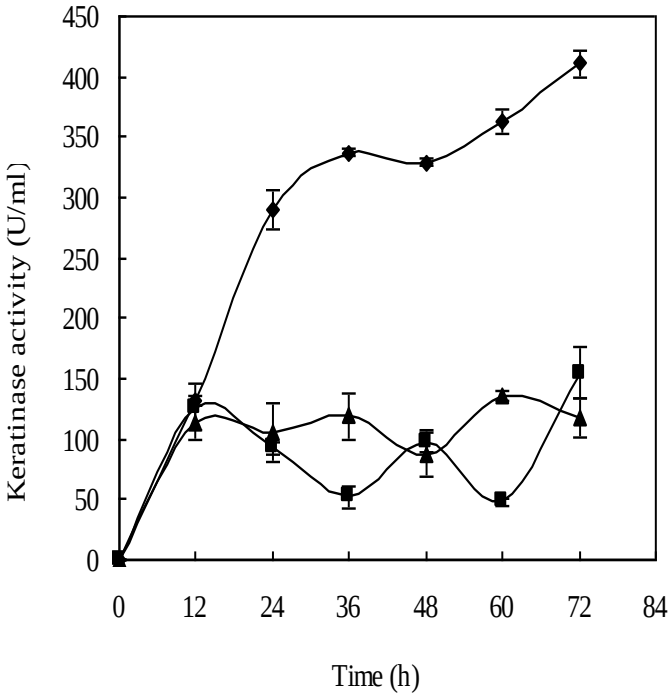

Figure 4. Effect of three different substrates on keratinase activity from *Bacillus* sp. 50-3. To measure activity, the enzyme was assayed at each 12 h for 72 h by the method in above. (closed diamond) native chicken feather; (closed triangle) cattle tendon; (closed square) human hair. Each point represents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

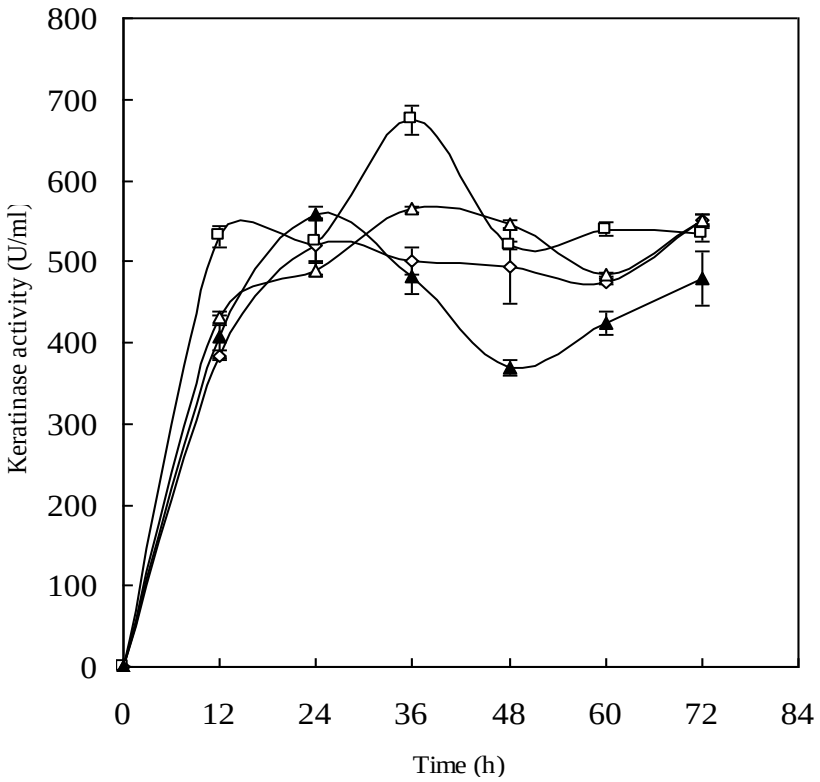

Figure 5. Effect of different feather concentration on the keratinase activity from *Bacillus* sp. 50-3. To measure activity, the enzyme was assayed at each 12 h for 72 h by the method in above. Open diamond 0.5%, open sequare 1.0%, open triangle 1.5%, closed triangle 2.0%. Each point represents the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

Effect of different feather meal concentration

The effect of different feather meal concentrations on keratinase activity was investigated. The results showed no significant importance of the feather meal concentration on the keratinase activity (Figure 5). The optimum feather meal concentration was determined as 1.0% (w/v) as the keratinase activity was relatively high.

Discussion

In early reports, the spore-forming bacterial strains have been isolated from variously ecosystems such as soil, water, compost, and food [8, 9, 15, 30, 31]. However, the isolates from animal feces were less reported [32-34]. In this study, many spore-forming bacteria were found in animal feces, which is an organic matter-rich environment. The most active strain 50-3 was isolated from Calotes versicolor (an agamid lizard) feces, which could be as a microorganism of environmental origin. It is well known that Calotes versicolor is an agamid lizard found widely in Asia with the main food of insects such as crickets, beetles, and spiders. Therefore, it could be some microorganisms colonizing in its gastrointestinal tract to degrade the insects' scute, which is full of keratin, and facilitate efficient digestion [34-36].

The morphological and physiological characteristics and the 16S rRNA sequence result indicated that strain 50-3 was within the *Bacillus subtilis* group. In a previous study on taxonomy, species included in the *B. subtilis* group are the following: *B. velezensis*, *B. atrophaeus*, *B. mojavensis*, *B. malacitensis*, *B. axarquiensis*, *B.*

nematocida, *B. vallismortis*, *B. subtilis*, and *B. amyloliquefaciens* [22, 29, 37, 38]. The 16S rDNA sequence result demonstrated that the strain 50-3 had high similarity (>99%) to the strains of *B. velezensis*, *B. vallismortis*, and *B. subtilis*. However, although a level of similarity which was greater than the borderline 98.7% may be used to define bacterial genomic species [39-41], only the 16S rDNA sequencing was not enough in determining which species the strain 50-3 belonged to. Further studies are necessary for its accurate identification. Therefore, the strain 50-3 was temporary named as *Bacillus sp*. 50-3 at present.

The isolated *Bacillus sp*. 50-3 is a novel *Bacillus* strain that can degrade feather keratin with high keratinase activity. Compared to many other feather degrading strains [14, 16, 30, 42, 43], it can degrade the native feather in much shorter time (36 h) and has a relatively high keratinase activity (680 ± 25 U/ml) at the same conditions comparing to other feather degrading strains.

The strain *Bacillus sp*. 50-3 can grow at the wide ranges of temperatures and pH levels. During cultivation, no matter the initial pH was low (pH 4.0) or high (pH 11.0) in feather meal medium, the final pH would come to around 8.0, which indicated the keratin hydrolysis [23, 44]. At low initial pH, it demonstrated the same result as the previous reports [16, 23, 44]. However, at high initial pH, the pH would decrease first, which might be caused by the keratin degradation and the free carboxyl increase in the medium to balance the pH for proper bacterial strain growth.

Keratin is an abundant structural protein in feather and hair [4, 15], and an exogenous inducer of keratinase [9, 42]. The keratinase can be largely induced in feather medium with the higher enzyme activity than that in hair medium (Figure 4). The hair is hard to be degraded because hair keratin has much more α-helix structures that are difficulty to be degraded than that in feather keratin. On the other hand, the cattle tendon has collagen as its dominant protein. Therefore, the keratinase activity was very low even if the cattle tendon was degraded eventually. This result was different from the previous reported *F. islandicum* AW-1 [14].

The newly isolated strain 50-3 was a new *Bacillus* strain with high keratinase activity and unorthodox sources that indicated its novel characters. The strain can degrade the native feather in much shorter time in mild reactive conditions, which suggested its potential applications and efficiency in biotechnological processes involving keratin hydrolysis. In addition, the animal feces are organic matter-rich environment, which may lead to isolate more useful microorganisms from them.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (U1404332); The Science and Technology Innovation Talent Project in Henan Province Colleges and Universities, China (16HASTIT017); The Project of Science and Technology Innovation Team of the Henan University of Animal Husbandry and Economy (HUAHE2015015).

Reference

1. Kumar P, Sharma SM, 2016. Enzymes in green chemistry: The need for environment and sustainability. Int J Appl Res. 2(6): 337-341.
2. Verma A, Singh H, Anwar S, Chattopadhyay A, Tiwari KK, Kaur S, Dhilon GS. 2016. Microbial keratinases: industrial enzymes with waste management potential. Crit Rev Biotechnol. 37(4): 1-16.
3. Marcus S, Ajay S, Owen PW. 2004. Developments in the use of Bacillus species for industrial production. Can J Microbiol. 50(1): 1-17.
4. Wang B, Yang W, McKittrick J, Meyers MA. 2016. Keratin: Structure, mechanical properties, occurrence in biological organisms, and efforts at bioinspiration. Prog Mater Sci. 76(3): 229-318.
5. Kim JM, Lim WJ, Suh HJ. 2001. Feather-degrading Bacillus species from poultry waste. Process Biochem. 37(3): 287-291.
6. Williams CM, Richter CS, MacKenzie JM, Shih JCH. 1990. Isolation, identification, and characterization of a feather-degrading bacterium. Appl Environ Microbiol. 56: 1509-1515.

7. Ferrareze PAG, Correa APF, Brandelli A. 2016. Purification and characterization of a keratinolytic protease produced by probiotic Bacillus subtilis. Biocatal Agr Biotechnol. 7: 102-109.
8. Fellahi S, Chibani A, Feuk-Lagerstedt E, Taherzadeh MJ, 2016. Identification of two new keratinolytic proteases from a Bacillus pumilus strain using protein analysis and gene sequencing. AMB Express. 6(1): 42.
9. Sanghvi G, Patel H, Vaishnav D, Oza T, Dave G, Kunjadia P, Sheth, N. 2016. A novel alkaline keratinase from Bacillus subtilis DP1 with potential utility in cosmetic formulation. Int J Biol Macromol. 87: 256-262.
10. Chao YP, Xie FH, Yang J, Liu JH, Qian SJ. 2007. Screening for a new streptomyces strain capable of efficient keratin degradation. J Environ Sci. 19(9): 1125-1128.
11. Gradisar H, Kern S, Friedrich J. 2000. Keratinase of Doratomyces microsporus. Appl Microbiol Biot. 53(2): 196-200.
12. Sousa M, Souza O, Maciel M, Cruz R, Rêgo MG, Magalhães O, Pessoa-Júnior A, Porto A, Souza-Motta C. 2015. Keratinolytic potential of fungi isolated from soil preserved at the Micoteca URM. Eur J Biotechnol Biosci. 3: 10-15.
13. Bernal C, Cairo J, Coello N. 2006. Purification and characterization of a novel exocellular keratinase from Kocuria rosea. Enzyme Microb Tech. 38(1): 49-54.
14. Nam GW, Lee D W, Lee HS, Lee NJ, Kim BC, Choe E A, Hwang J K, Suhartono MT, Pyun YR. 2002. Native-feather degradation by Fervidobacterium islandicum AW-1, a newly isolated keratinase-producing thermophilic anaerobe. Arch Microbiol. 178(6): 538-547.
15. Riessen S, Antranikian G. 2001. Isolation of Thermoanaerobacter keratinophilus sp. nov., a novel thermophilic anaerobic bacterium with kertinolytic activity. Extremophiles. 5(6): 399-408.
16. Riffel A, Lucas F, Heeb F, Brandelli A. 2003. Characterization of a new keratinolytic bacterium that completely degrades native feather keratin. Arch Microbiol. 179(4): 258-265.
17. Thys RCS, Lucas FS, Riffel A, Heeb P, Brandelli A. 2004. Characterization of a protease of a feather-degrading Microbacterium species. Lett Appl Microbiol. 39(2): 181-186.
18. Wang LY, Cheng GY, Ren YX, Dai Z, Zhao ZS, Liu F, Li SY, Wei YH, Xiong J, Tang XF, Tang B. 2015. Degradation of intact chicken feathers by Thermoactinomyces sp. CDF and characterization of its keratinolytic protease. Appl Microbiol Biot. 99(9): 3949-3959.
19. de Oliveira CT, Pellenz L, Pereira JQ, Brandelli A, Daroit DJ. 2016. Screening of Bacteria for Protease Production and Feather Degradation. Waste Biomass Valo. 7(3): 447-453.
20. Onifade AA, Al-Sane NA, Al-Musallam AA, Al-Zarban S. 1998. Potentials for biotechnological applications of keratin-degrading microorganisms and their enzymes for nutritional improvement of feathers and other keratins as livestock feed resources. Bioresource Techno. 66(1): 1–11.
21. Shih JCH. 1993. Recent development in poultry waste digestion and feather utilization—a review. Poultry Sci. 72(9): 1617-1620.
22. Wang HY, Liu DM, Liu Y, Cheng CF, Ma QY, Huang Q, Zhang Y Z. 2007. Screening and mutagenesis of a novel Bacillus pumilus strain producing alkaline protease for dehairing. Lett Appl Microbiol. 44(1): 1-6.
23. Sangali S, Brandelli A. 2000. Feather keratin hydrolysis by a Vibrio sp. strain kr2. J Appl Microbiol. 89(5): 735-743.
24. Kersters K, Vancanneyt M: Bergey's Manual of Systematic Bacteriology. Berlin: Springer Verlag; 2005: 21-53.
25. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG, 2007. Clustal W and Clustal X version 2.0. Bioinformatics. 23(21): 2947-2948.
26. Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 4(4): 406-425.
27. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood. Mol Biol Evol. 28(10): 2731-2739.
28. Cristina RG, Victoria B, Fernando MC, Inmaculada L, Emilia Q. 2005. Bacillus velezensis sp. nov., a surfactant-producing bacterium isolated from the river Ve´ lez in Ma´ laga, southern Spain. Int J Syst Evol Micr. 55(1): 191-195.
29. Roberts MS, Nakamura LK, Cohan FM. 1996. Bacillus vallismortis sp. nov., a close relative of Bacillus subtilis, isolated from soil in Death Valley, California. Int J Syst Bacteriol. 46(2): 470-475.
30. Lin X, Inglis GD, Yanke LJ, Cheng KJ. 1999. Selection and characterization of feather-degrading bacteria from canola meal compost. J Ind Microbiol Biot. 23(2): 149-153.
31. Shih IL, Wu PJ, Shieh CJ. 2005. Microbial production of a poly (g-glutamic acid) derivative by Bacillus subtilis. Process Biochem. 40(8): 2827-2832.
32. Nakada Y, Ohta Y. 1999. Purification and Properties of Hydrogen Sulfide Oxidase from Bacillus sp. BN53-1. J Biosci Bioeng. 87(4): 452-455.
33. Swiecicka I, Fiedoruk K, Bednarz G. 2002. The occurrence and properties of Bacillus thuringiensis isolated from free-living animals. Lett Appl Microbiol. 34(3): 194-198.
34. Meng JY, Xing LL, Zhao XJ. 2013. Isolation of a feather-degrading Bacillus subtilis strain from the alimentary tract of grebes. Afr J Microbiol Res. 7(21): 2550-2559.
35. Angelis MD, Siragusa S, Berloco M, Caputo L, Settanni L, Alfonsi G, Amerio M, Grandi A, Ragni A, Gobbetti M. 2006. Selection of potential probiotic lactobacilli from pig feces to be used as additives in pelleted feeding. Res Microbiol. 157(8): 792-801.
36. Bevilacqua L, Ovidi M, Mattia ED, Trovatelli LD, Canganella F. 2003. Screening of Bifidobacterium strains isolated from human faeces for antagonistic activities against potentially bacterial pathogens. Microbiol Res. 158(2): 179-185.
37. Roberts MS, Nakamura LK, Cohan FM. 1994. Bacillus mojavensis sp. nov., distinguishable from Bacillus subtilis by sexual isolation, divergence in DNA sequence, and differences in fatty acid composition. Int J Syst Evol Micr. 44(2): 256-264.
38. Thorsen L, Kandob CK, Sawadogo H, Larsen N, Diawara B, Ouédraogo GA, Hendriksend NB, Jespersena L. 2015. Characteristics and phylogeny of Bacillus cereus strains isolated from Maari, a traditional West African food condiment. Int J Food Microbiol. 196: 70-78.

39. Stackebrandt E, Ebers J. 2006. Taxonomic parameters revisited: tarnished gold standards. Micr Today. 6: 152-155.
40. Stackebrandt E, Goebel BM. 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Evol Micr. 44(4): 846-849.
41. Poli A, Guven K, Romano I, Pirinccioglu H, Guven RG, Euzeby JPM, Matpan F, Acer O, Orlando P, Nicolaus B. 2012. Geobacillus subterraneus subsp. aromaticivorans subsp. nov., a novel thermophilic and alkaliphilic bacterium isolated from a hot spring in Sırnak, Turkey. J Gen Appl Microbiol. 58(6): 437-446.
42. El-Refai HA, AbdelNaby MA, Gaballa A, El-Araby MH, Fattah AFA. 2005. Improvement of the newly isolated Bacillus pumilus FH9 keratinolytic activity. Process Biochem. 40(7): 2325-2332.
43. Okoroma EA, Garelick H, Abiola OO, Purchase D. 2012. Identification and characterisation of a Bacillus licheniformis strain with profound keratinase activity for degradation of melanised feather. Int Biodeter Biodegr. 74: 54-60.
44. Bach E, Lopes FC, Brandelli A. 2015. Biodegradation of α and β-keratins by Gram-negative bacteria. Int Biodeter Biodegr. 104: 136-141.