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Characterization of an extreme alkaline-stable keratinase from the draft genome of feather-degrading *Bacillus* sp. JM7 from deep-sea

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Abstract

Bacillus sp. JM7, a strain isolated from the deep-sea of the South China Sea, was found to efficiently degrade 79.4% native chicken feather within 30 h. Scanning electron microscopy analysis showed that JM7 strain could gradually degrade feather by modifying the microstructure of feather keratin. A total of 25 protease genes were predicted from the draft genome of JM7 strain, among which a predicted subtilisin-like serine protease (designated as Ker02562) was further characterized for its keratinolytic activity. The recombinant Ker02562 functioned at a wide range of temperatures from 30°C to 60°C, with an optimum at 40–50°C. Ker02562 was highly active at various pHs ranging from 5.0 to 13.0, with a maximum activity observed at pH 7.0–9.0. Remarkably, recombinant Ker02562 was stable in extreme alkaline environments (pH 10–13), which was much better than most other reported keratinases. Collectively, these favorable properties could make *Bacillus* sp. JM7 and Ker02562 attractive to be applied in the detergent formulation and feather bioconversion.

Key words: *Bacillus*, deep-sea, feather-degradation, keratinases, feather bioconversion, alkaline-stable

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1 Introduction

In the light of Food and Agriculture Organization of the United Nations, the generation of chicken feather waste can be estimated to be around 5 million tons every year (Forgács et al., 2013). Chicken feather is composed of 90%–92% keratin, which is a fibrous and insoluble structural protein rich in β-helical coils linked through disulfide bridges (da Gioppo et al., 2009). This renders them resistant to degradation by proteases such as pepsin, papain, and trypsin and thus causes serious environmental problems. Conventionally, feather wastes are currently utilized as animal feedstuffs after converted to feather meals by hydrothermal processing (Tiwary and Gupta, 2010). However, this process yields a product with poor digestibility, and causes the loss of heat sensitive nutritionally essential amino acids such as lysine, methionine and tryptophan, and the addition of non-nutritive amino acids such as lanthionine and lysinoalanine (Brandelli and Riffel, 2005).

Due to the growing interest in preventing pollution, biodegradation of feather keratin by microorganisms possessing keratinolytic activity represents an alternative attractive treatment technology for poultry waste (Jeong et al., 2010). Diverse microorganisms have been reported to utilize feather keratin, including bacteria, fungi and actinomycetes. For example, *Bacillus* sp. CH-1 isolated from the gut of the tarantula *Chilobrachy sguangxiensis* was able to efficiently degrade the intact feather under the action of four kinds of key protease simultaneously (Liu et al., 2014). A feather-degrading fungi *Aspergillus fumigatus* TKF1 was isolated from soil, which could degrade feather and lead to an increase in free amino acids such as cysteine, threonine, phenylalanine, leucine, valine, and isoleucine (Paul et al., 2014). A thermostable extracellular keratinase (KERAK-29) was purified with a high production of 24 000 U/mL from a thermophilic actinomycete strain Cpt29 which was isolated from Algerian poultry compost (Habbeche et al., 2014).

In general, keratinases are regarded as the crucial enzymes for degrading chicken feathers. Nowadays, most keratinases, especially keratinases from *Bacillus* strains, are also classified into serine proteases due to their 97% sequence homology with alkaline protease and their activity inhibition by the same inhibitors that inhibit serine proteases (Zaghloul, 1998; Bressollier et al., 1999). Keratinolytic microorganisms and microbial keratinases could be interesting for a wide spectrum of industries, as they can find applications in the detergent, pharmaceutical, animal feed, cosmetic and fertilizer industries, as well as in leather processing

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and keratinous wastes bioconversion (Brandelli, 2008; Syed et al., 2009; Brandelli et al., 2010; Wang et al., 2011). Additionally, keratinases that cleave β-pleated structure of feather keratin have been reported to dissolve prion plaques, thus may serve as decontaminating materials for prion degradation (Gupta et al., 2013). To this date, many keratinolytic microorganisms including *Bacillus* species have been found to produce keratinase. However, to our best knowledge, no keratinolytic bacteria or keratinase has been isolated from the deep-sea environments. In this study, a chicken-feather-degrading strain *Bacillus* sp. JM7, was isolated from the deep sea water of the South China Sea at the depth of 2 000 m. The feather-degrading activity of JM7 strain as well as keratinase protein responsible for feather degradation was characterized.

2 Materials and methods

2.1 *Bacterial strain and profile of chicken feather degradation*

Bacillus sp. JM7 was isolated from seawater sample of the South China Sea (21°03′N, 118°23′E) at the depth of 2 000 m, and was deposited at the Marine Culture Collection of China (Accession number: MCCC 1A10889) and China Center for Type Culture Collection (Accession number: CCTCC M2015179). Notably, when grown in an optimized culture medium ($K_2HPO_4 \cdot 3H_2O$ 1 g/L, $MgCl_2 \cdot 6H_2O$ 0.2 g/L, $Na_2HPO_4 \cdot 12H_2O$ 12 g/L, 0.3% (w/v) chicken feather) at 40°C, *Bacillus* sp. JM7 efficiently degraded native chicken feather (Fig. 1a). For the characterization of the JM7 feather degradation profile, an overnight culture of JM7 strain (grown in Luria–Bertani media, OD_{600}=2) was inoculated (6%, v/v) to the optimized culture medium and was further cultured at 40°C with shaking. At different intervals post JM7 strain inoculation (0, 10, 25, 30, 48, 60, and 72 min post addition), the degraded feather residue was collected by filtration and was dried in an oven at 60°C for 24 h to constant weight. The dry weight of feather residue was obtained gravimetrically, and the degradation rate was calculated according to the following formula:

$$\text{degradation rate} = \frac{\text{dry weight of added feather} - \text{dry weight of feather residue}}{\text{dry weight of added feather}} \times 100\%.$$

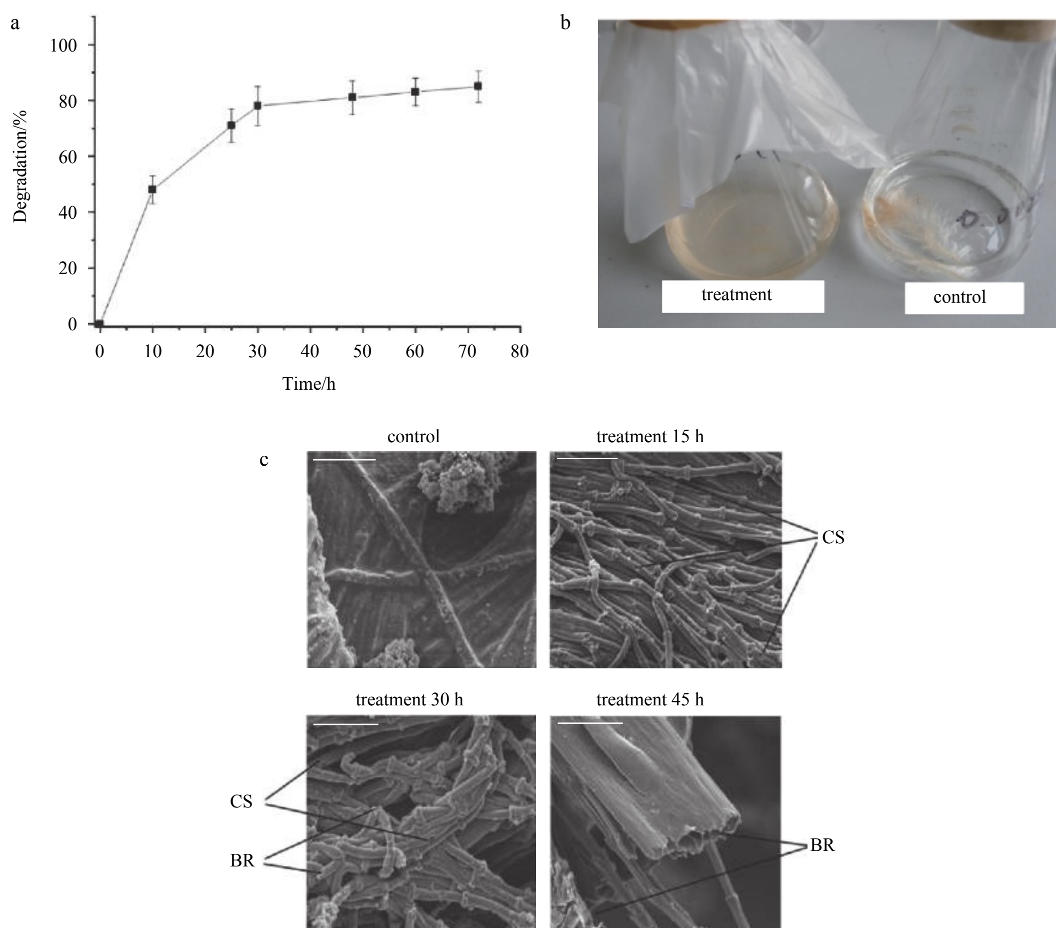

Fig. 1. Feather degradation profile of *Bacillus* sp. strain JM7. a. Time-course profile of feather degradation by *Bacillus* sp. strain JM7. Dry weight of feather residue was measured at different intervals post JM7 strain addition, and the feather degradation rate was further calculated. Values are expressed as mean of triplicate independent assays with standard deviation. b. Feather degradation by *Bacillus* sp. strain JM7. Chicken feathers were treated with (left) or without (right) strain JM7. c. Scanning electron micrographs of degraded chicken feathers during the growth of *Bacillus* sp. strain JM7. The SEM images of chicken feathers with (treatments) or without (control) JM7 degradation were presented. CS and BR indicate the cracked surface and break-down site of feather keratin, respectively.

2.2 *Scanning electron microscopy (SEM) analysis*

For scanning electron microscopy analysis, the degraded feather residue was harvested by filtration with filter paper, and was dried and fixed with glutaraldehyde solution (2.5% (v/v) in 50 mmol/L Tris-HCl (pH 7.5) with 3% (w/v) NaCl) at 75°C for 30 min. Subsequently, the fixed feather was dehydrated in a graded ethanol series and immersed in *t*-butyl alcohol, followed by drying using a freeze-drying method, and coating with gold under vacuum (Goldstein et al., 1981). The microstructure of feather was examined under a FEI Quanta 450 scanning electron microscope (USA) at an accelerating voltage of 10 kV.

2.3 *Bacillus sp. JM7 genome sequencing and gene prediction*

The draft genome of *Bacillus* sp. JM7 was sequenced by illumina Solexa High-Seq 2000 paired-end sequencing technology in BGI (China). The reads were assembled using SOAP *de novo* software version 1.05 (Li et al., 2008). Protein-coding sequences were predicted by the Glimmer 3.0 program (Delcher et al., 2007) and annotated by BLAST searches against the database of non-redundant protein sequences from NCBI, Swiss-Prot, TrEMBL, COG and KEGG. Ribosomal RNA genes and transfer RNAs were detected using RNAmmer 1.2 software (Lagesen et al., 2007) and tRNAscan-SE (Schattner et al., 2005), respectively. The draft genome sequence of *Bacillus* sp. JM7 is available at DDBJ/EMBL/GenBank under the accession JXZC00000000. The version described in this paper is version JXZC01000000.

2.4 *Sequence analysis*

The BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) was used to analyze the sequence similarities of Ker02562. The conserved motif of Ker02562 was identified by searching against NCBI Conserved Domain Database based on sequence homology. MEGA Program (DNAstar, USA) was used to generate the phylogenetic tree according to the neighbor-joining method.

2.5 *Overexpression and purification of recombinant Ker02562*

The *ker02562* gene was amplified from the genomic DNA of *Bacillus* sp. JM7 with an upstream primer (5′-GCGGAGCTCATGCAAGGTGAAATTAG-3′) and a downstream primer (5′-GCGAAGCTTTCACCCAATCTGAGCAAGC-3′), which contained the restriction site for *Sac* I and *Hind* III (underlined), respectively. The amplicon was then inserted into pColdI expression vector (Biovector, China) downstream of the 6×histine tag after digested with *Sac* I and *Hind* III. For the overexpression and purification of 6×histine-tagged recombinant Ker02562 protein, the recombinant pColdI-*ker02562* vector as well as the empty pColdI vector were transformed into *E. coli* BL21(DE3) cells, separately. The recombinant *E. coli*-pColdI and *E. coli*- pColdI-*ker02562* cells were then grown at 37°C in LB medium containing 100 μg/mL ampicillin, and were induced with 1 mmol/L isopropyl-β-D-thiogalactopyranoside (IPTG) for additional 12 h at 16°C when the OD_{600} of the cultures reached 0.5–0.6. The bacteria were harvested by centrifugation at 15 000 *g* for 10 min at 4°C and were disrupted by sonication for 30 min at a pulse frequency of 3 s/3 s. After the separation of soluble and insoluble components of cell extract by centrifugation for 15 min at 15 000 *g*, the soluble supernatant was collected and the 6×histine-tagged recombinant Ker02562 was then purified with a Ni-NTA affinity column according to the manufacturer's recommendations (Qiagen, Germany). The purified Ker02562 protein was resolved by glycine-SDS-PAGE and visualized by Coomassie brilliant blue staining.

2.6 *Enzyme activity assay*

Generally, the enzymatic activity of Ker02562 was measured using casein as substrates according to the modified method of Nam et al. (2002). Briefly, 400 μL diluted enzyme was mixed with 400 μL preheated 0.25% (w/v) casein in 10 mmol/L Tris-HCl buffer (pH 9.0), and was incubated at 40°C for 10 min. Then the enzymatic reaction was stopped by addition of 800 μL 0.4 mol/L trichloroacetic acid solution (TCA), followed by centrifugation at 15 000 *g* for 10 min at 4°C. The absorbance of the supernatant was measured spectrophotometrically at the wavelength of 280 nm. One unit of enzymatic activity was defined as the 0.01 increase of absorbance at 280 nm/min under the described conditions. The enzymatic activity of Ker02562 towards feather powder was determined using soluble feather powder (0.2%, w/v) as substrates instead of casein with the same method described above.

2.7 *Detection of free amino acids in feather powder enzymatic degradation culture*

A total of 400 μL diluted enzyme was mixed with 0.30% (w/v) feather powder, and incubated at 40°C for 30 min. In order to determine the components and contents of free amino acids produced from enzymatic hydrolysis, the supernatant of reaction mixture and the mixed amino acids standard were analyzed under the same conditions with an anion exchange chromatograph (DIONEX, Sunnyvale, CA, USA) equipped with a 250 mm×4 mm IonPac column (ASII-HC). The mobile phase consisted of 200 mmol/L NaOH in water (Solvent A), 600 mmol/L NaAc in water (Solvent B) and pure water (Solvent C). After the sample was loaded, the column was gradient washed at a flow rate of 0.25 mL/min for 50 min as followed: 20% A, 0% B, 12 min; 20%–32% A, 0% B, 4 min; 32%–24% A, 0%–40% B, 8 min; 24% A, 40% B, 16 min; 20% A, 0% B, 10 min. The liquid chromatography (LC) plot was acquired by monitoring the electrical conductivity of the eluent. The peaks were identified by comparing the retention times with those of standards, and the relative quantification was achieved by comparing the peak areas.

2.8 *Characterization of recombinant Ker02562*

The temperature effects on Ker02562 activity was studied by detecting the enzyme activity at various temperatures ranging from 20°C to 80°C in 50 mmol/L Tris-HCl buffer (pH 8.0). The thermostability of recombinant Ker02562 was evaluated by monitoring the residual enzyme activity after incubating the enzyme in 50 mmol/L Tris-HCl buffer (pH 8.0) in the absence of substrate at different temperatures (20, 40, 50, 60 and 80°C) for various periods (0, 5, 10, 20, 30 and 60 min).

The pH effects on Ker02562 activity was investigated by incubating Ker02562 with substrates at 40°C in the following buffers: 50 mmol/L Na_2HPO_4/citric acid solution (pH 5.0–8.0), 50 mmol/L Tris-HCl buffer (pH 7.0–9.0), or 50 mmol/L Gly/NaOH buffer (pH 9.0–13.0) (Gao et al., 2015; Zhou et al., 2015). For the determination of Ker02562 pH stability, the enzyme was incubated at 40°C in pH 5.0–13.0 solutions for 2 h prior to activity detection.

The effects of various additives including metal ions and chemical reagents were assessed by pre-incubation of Ker02562 with various metal ions and chemical reagents of different final concentrations (1, 5 mmol/L or 1%, 5%) at 40°C and pH 8.0, followed by residual activity determination under the standard conditions as described above. The agents used in this study were as followed: metal ions (Cs^+, Ni^{2+}, Fe^{2+}, Co^{2+}, Cd^{2+}, Sr^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, K^+, Na^+, Fe^{3+}, Mg^{2+} and Zn^{2+}), and chemical reagents (PMSF, EDTA, DMSO, isopropanol and acetonitrile).

3 Results

3.1 *Characterization of a feather-degrading strain Bacillus sp. JM7*

A chicken-feather-degrading strain JM7 was isolated from the deep-sea water of the South China Sea at the depth of 2 000 m. The 16S rRNA sequence of JM7 (GenBank accession number CL137909) shared 99% identity with the 16S rRNA gene sequence of *Bacillus aquimaris* strain TF-12, which allowed its identification as *Bacillus* sp. JM7. Notably, JM7 strain was found to degrade chicken feather efficiently. As shown in Fig. 1a, when grown in an optimized culture medium it could rapidly degrade 79.4% feather within 30 h, which was significantly higher than most of other reported microorganisms capable of feather keratin degradation. After 48 h of JM7 strain culture, the feathers in the broth culture completely fall off from scapus and was further hydrolyzed, leading to the change of the culture color to yellow (Fig. 1b). The effect of JM7 strain on chicken feather microstructure was examined by scanning electron microscopy. As shown in Fig. 1c, the keratins in the controls were intact and maintained a tight fabric structure. However, after treated with JM7 strain for 15 h, the surface of keratin was cracked, and was more obviously to a greater extent at 30 h. The feather began to break down at 30 h, and was destroyed at 45 h after treatment (Fig. 1c), showing the extraordinary ability of JM7 strain to degrade native chicken feather.

3.2 *Discovery of Bacillus sp. JM7 genes involved in feather degradation*

To find the genes of *Bacillus* sp. JM7 that may involve in feather degradation, whole genome shot-gun sequencing was performed using illumina Solexa High-Seq 2000 platform. The *Bacillus* sp. strain JM7 genome featured 4 271 predicted ORFs, including 3 945 genes (92.36%) encoding known-function proteins, 1 166 (27.3%) genes encoding hypothetical proteins. In addition, 326 (7.63%) genes had no matches against non-redundant protein sequence database. There were 225 genes related to protein metabolism and 468 genes related to the metabolism of amino acids and derivatives, when the contigs were submitted to RAST annotation server. Based on annotation, a total of 25 predicted protease genes were found in the *Bacillus* sp. strain JM7 genome (Table 1). Since most of keratinases belong to serine alkaline protease (Zaghloul, 1998; Bressollier et al., 1999), some of the predicted serine proteases in *Bacillus* sp. JM7 genome may involve in the feather-degrading process. To support this hypothesis, 02562_1 protein (designated as Ker02562), a predicted subtilisin-like serine protease that shared 98% similarity to known serine protease (Table 1), was further characterized.

3.3 *Sequence analysis of Ker02562*

Nucleotide Blast result revealed that DNA sequence of *ker02562* had no significant similarity with the sequence of any known gene (<85% similarity). The amino acid sequence of Ker02562 shared 98%, 94%, 93% and 85% identity with serine proteases from *Bacillus aquimaris* (gi|764369150), *Bacillus vietnamensis* (gi|736757371), *Bacillus vallismortis* (gi|452056156) and *Bacillus enclensis* (gi|960407090), respectively. However, these top matched serine proteases were all predicted from the whole genome sequence, and no previous study has characterized these enzymes. As shown in Fig. 2, six conserved active sites of peptidase superfamily (Asp50, His87, Ile131, Leu150, Asn179 and Ser246) were identified in the amino acid sequence of Ker02562, which were crucial for the catalysis of serine proteases.

According to catalysis sites and inhibitor types, proteases can be classified into four subgroups, namely serine proteases, aspartate proteases, cysteine proteases and metal proteases. To de-

Table 1. Predicted protease of *Bacillus* sp. JM7

Query protein name	Query protein length	Description	Protein similarity/%
01965_1	171	intracellular protease, PfpI family	92
00873_1	470	Clp protease ATPase	92
00800_1	424	zinc protease	84
00193_1	291	serine protease	85
03156_1	543	serine protease	68
02217_1	416	zinc protease	50
03838_1	799	protease	91
04139_1	288	zinc metalloprotease	87
02947_1	552	Lon protease	90
04206_1	231	putative chaperone or protease	79
00666_1	404	alkaline serine protease	90
03215_1	708	ATP-dependent Clp protease ATP-binding protein	82
02562_1	320	subtilisin-like serine protease	98
03296_1	193	Clp protease	96
00817_1	413	zinc protease	92
00799_1	429	zinc protease	90
03760_1	413	serine protease	87
04224_1	736	protease, transglutaminase superfamily protein	74
03079_1	369	germination protease	89
04023_1	815	ATP-dependent Clp protease ATP-binding protein	97
02385_1	235	zinc protease	86
00365_1	376	serine protease	76
03048_1	425	protease	94
00004_1	267	protease	82
04019_1	423	ATP-dependent protease	96

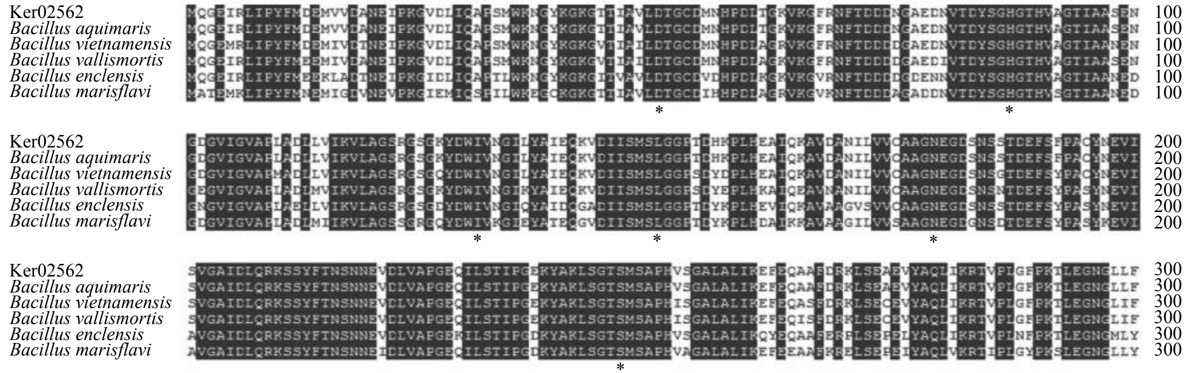

Fig. 2. Multiple alignment of amino acids sequences of Ker02562 and serine proteases of other bacteria. The serine proteases aligned are from *Bacillus aquimaris* (gi|764369150), *Bacillus vietnamensis* (gi|736757371), *Bacillus vallismortis* (gi|452056156), *Bacillus enclensis* (gi|960407090) and *Bacillus marisflavi* (gi|850298422). The identical residues of all aligned proteins are shaded black. The six conserved active sites are indicated with asterisks.

termine the subfamily of Ker02562, amino acid sequences of representative proteases belonging to four different groups were aligned and a phylogenetic tree was further generated to compare the amino acid sequence homology. The data showed that Ker02562 can be classified into serine protease, as it clustered with representative serine proteases in the phylogenetic tree (Fig. 3)

3.4 *Heterologous expression and purification of recombinant Ker02562 protein*

The 963 bp length *ker02562* gene was amplified from the genome of *Bacillus* sp. JM7, then was cloned into the pColdI expression vector and heterologously overexpressed in the *E. coli* BL21 (DE3) cells as an N-terminally His-tagged recombinant protein. As shown in Fig. 4a, the induced *E.coli*-pColdI-*ker02562* cultures

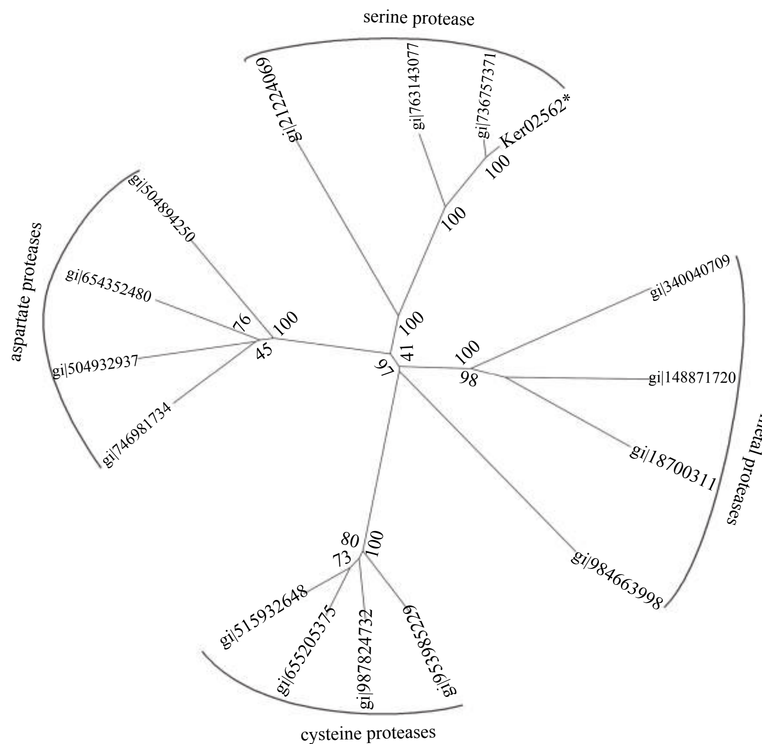

Fig. 3. Phylogenetic tree of proteases based on amino acid sequence homology. The phylogenetic tree was constructed using Mega 5.1 software according to the neighbour-joining method. Numbers alongside the tree branch present the bootstrap values of 1 000 trials. The scale bar indicates ten nucleotide substitutions per hundred nucleotides. The asterisk symbol indicates the position of Ker06562 in the tree. The analyzed proteases were from *Anabaena* sp. 90 (gi|504894250), *Hassallia byssoidea* VB512170 (gi|746981734), *Rivularia* sp. PCC 7116 (gi|504932937), *Mastigocoleus testarum* (gi|654352480), *Pseudomonas resinovorans* (gi|655205375), *Pseudomonas mendocina* (gi|515932648), *Pseudomonas citronellolis* (gi|953985229), *Pseudomonas aeruginosa* (gi|987824732), *Pseudomonas fluorescens* (gi|984663998), *Vibrio campbellii* HY01 (gi|148871720), *Pseudoalteromonas* sp. A28 (gi|18700311), *Vibrio cholerae* HE39 (gi|340040709), *Streptomyces coelicolor* A3 (gi|21224069), *Bacillus vietnamensis* (gi|736757371), and *Bacillus* sp. SG-1 (gi|763143077).

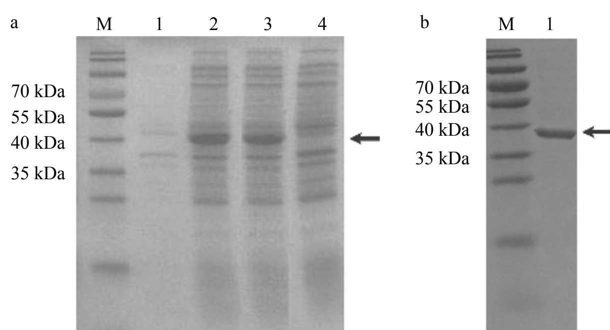

Fig. 4. Heterologous overexpression (a) and purification (b) of recombinant Ker02562. a. Lane M represents protein marker; Lane 1 *E. coli*-pColdI-*ker02562* total cell extract, non-induced; Lane 2 *E. coli*-pColdI-*ker02562* total cell extract, induced; Lane 3 *E. coli*-pColdI-*ker06562* soluble cell extract, induced; and Lane 4 *E. coli*-pColdI-*ker02562* insoluble cell extract, induced. b. Lane M represents protein marker and Lane 1 purified recombinant Ker02562. The arrows indicate the protein band corresponding to recombinant Ker02562.

showed the existence of a new protein band in the SDS-PAGE gel with an approximate molecular weight of 38 kDa, which was corresponding to the size of the 6×His-tagged Ker02562 fusion protein (Fig. 4a, Lane 2). Besides, most of the expressed Ker02562 fusion proteins were presented in soluble cell extract of induced *E.coli*-pColdI-*ker02562* cells (Fig. 4a, Lane 3), suggesting the successful expression of soluble recombinant Ker02562 protein in the cytoplasm. The recombinant Ker02562 was further purified with a Ni^{+} Affinity column, and was revealed as a single band on the SDS-PAGE gel (Fig. 4b).

Purified recombinant Ker02562 was enzymatically active against casein and feather powder with a specific activity of 334.8 U/mg and 123.4 U/mg, respectively. Seventeen amino acid components were detected in the feather powder degradation culture, including asparaginic acid, threonine, serine, glutamic acid, glycine, alanine, valerian glycine, cystine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine, arginine and proline (Table 2), exhibiting a good feather degrading activity of Ker02562.

Table 2. Components and contents of amino acids produced from feather powder degradation by Ker02562

Component	Content/%
Asparaginic acid	0.93
Threonine	0.37
Serine	1.21
Glutamic acid	1.24
Glycine	0.78
Alanine	0.36
Cystine	0.25
Valerian glycine	0.55
Methionine	0.11
Isoleucine	0.27
Leucine	0.67
Tyrosine	0.36
Phenylalanine	0.97
Lysine	0.11
Histidine	0.09
Arginine	0.91
Proline	1.01

3.5 *Characterization of recombinant Ker02562 protein*

The effect of temperature on Ker02562 activity was investigated at a broad range of temperatures from 4°C to 80°C at pH 8.0 (Fig. 5a). Recombinant Ker02562 was active at the temperature range of 30°C to 60°C and was most active at 40–50°C. Notably, Ker02562 showed approximately 90% of its maximum activity at 50°C, suggesting that Ker02562 was able to adapt to moderate-high temperature environments (Fig. 5a). Additionally, Ker02562 was stable between 20°C and 50°C, and retained more than 80% and 65% of its maximum activity after incubation for 1 h at 40°C and 50°C respectively, displaying a good thermostability at moderate-high temperature environments (Fig. 5b). The pH profiles showed that recombinant Ker02562 was active at various pHs ranging from 5.0 to 13.0, with an optimum activity observed at pH 7.0–9.0. Remarkably, Ker02562 can adapt to extreme alkaline environment, as it still retained more than 45% of its maximum activity at pH 13.0 (Fig. 5c). Most importantly, nearly 80%, 60%, 45%, and 35% Ker02562 residual activity was detected after 1 h of incubation at pH 10, 11, 12 and 13, respectively, indicating that the recombinant enzyme was stable in extreme alkaline environments (Fig. 5d).

The effects of various metal ions and other chemical reagents on the activity of recombinant Ker02562 are summarized in Tables 3 and 4, respectively. Among the tested metal ions and chemical reagents, Sr^{2+}, Mg^{2+} and Ca^{2+}, especially Mg^{2+} and Ca^{2+}, can significantly enhance the Ker02562 activity (Table 3). In contrast, Ker06562 activity was strongly inhibited by several metal ions (Zn^{2+}, Cu^{2+}, Cd^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cs^{+}, Na^{+}) and chemical reagents (PMSF, EDTA, DMSO, isopropanol, acetonitrile) at high concentrations (5 mmol/L or 5%) (Tables 3 and 4). In addition, several metal ions including K^{+}, and Fe^{2+} exerted no obvious influences on Ker02562 activity (Table 3).

4 Discussion

Bacillus sp. JM7 showed a remarkable potential for the biodegradation of chicken feathers, as it could rapidly degrade 79.4% native chicken feather within 30 h, which was significantly higher than most of other reported keratinolytic microorganisms such as *Bacillus* sp. P7 (Corrêa et al., 2010), *Doratomyces microspores* (Gradišar et al., 2000) and so on. A total of 25 protease genes were predicted from the draft genome of *Bacillus* sp. strain JM7, and a predicted subtilisin-like serine protease, Ker02562, was further characterized. Although the purified Ker02562 was shown to be enzymatic active against casein and feather powder, further investigation for characterizing the remaining 24 predicted protease genes is warranted to depict the keratinolytic profile of *Bacillus* sp. JM7.

Ker02562 was active optimally at 40–50°C, and was stable in medium-high temperatures up to 50–60°C, which was similar with other keratinases from mesophilic microorganisms (Table 5). It was interesting that the keratinase isolated from the icy deep-sea environments could operate and be stable at temperatures up to 60°C. However, since no other keratinases has been reported from the deep-sea environments, the benefits of this thermotolerance of keratinases in the deep-sea environments remained unknown. Ker02562 showed an optimum pH at pH 7–9, and was active predominantly in the alkaline region of pH 7.0–13.0, which was similar with other reported keratinases (Table 5), since most of the reported keratinases were most active in neutral to alkaline environments ranging from 7.5–9.0 (Brandelli et al., 2010).

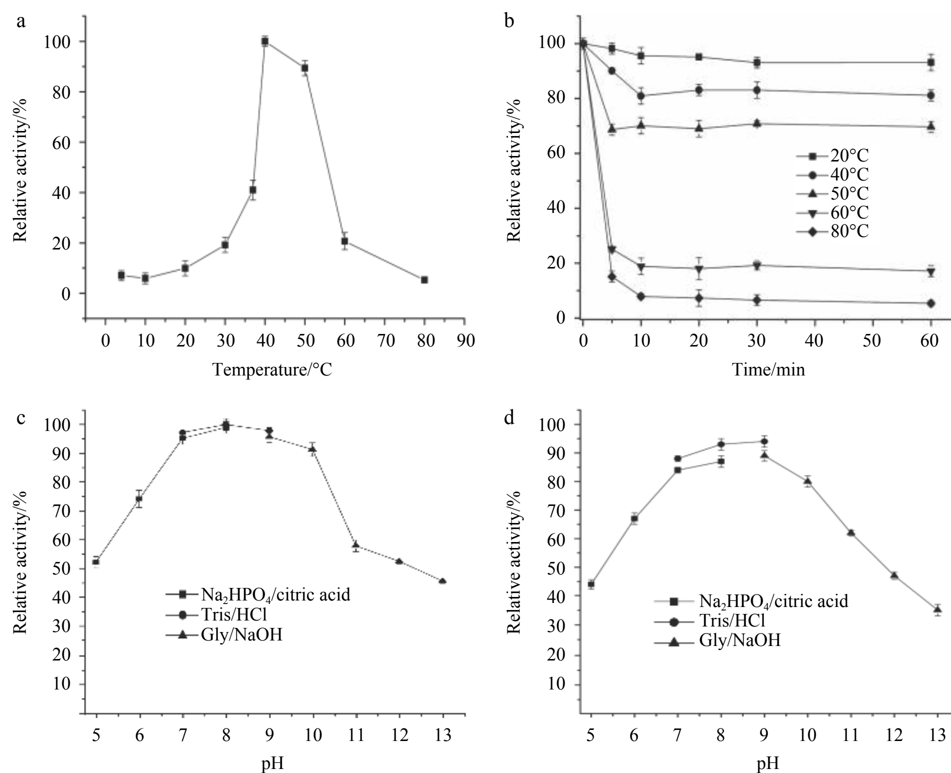

Fig. 5. Effects of temperature and pH on the activity and stability of recombinant Ker02562. a. Effect of temperature on the activity of recombinant Ker02562. The activity of Ker02562 was determined at a broad temperature range from 4°C to 80°C under standard assay conditions. b. Effect of temperature on the stability of recombinant Ker02562. The residual activity of Ker02562 was assayed after incubating Ker02562 in the absence of substrates at 20, 40, 50, 60 or 80°C for various time durations (0, 5, 10, 20, 30, 60 min). c. Effect of pH on the activity of recombinant Ker02562. The optimum pH of Ker02562 was determined by incubating Ker02562 in the following buffers prior to activity detection at 40°C: pH 5.0 to 8.0, 50 mmol/L Na_2HPO_4/citric acid solution; pH 7.0 to 9.0, 20 mmol/L Tris-HCl buffer; pH 9.0 to 13, 50 mmol/L Gly/NaOH buffer. d. Effect of pH on the stability of recombinant Ker02562. The residual activity of Ker02562 was determined after the pre-incubation of Ker02562 in buffers of desired pH (pH 5.0–13.0) at 40°C for 2 h. For all of the plots, values are presented as percentages of the maximum activity of Ker02562 (taken as 100%) and are expressed as mean of triplicate independent assays with standard deviation.

Table 3. Effects of metal ions and chemical reagents on Ker02562 activity

Metal ion	Relative activity/%		Metal ion	Relative activity/%	
	1 mmol/L	5 mmol/L		1 mmol/L	5 mmol/L
Control	100	100	Fe^{3+}	54	26
K^+	95	95	Co^{2+}	41.40	12.38
Na^+	89	80	Co^{2+}	19	12
Sr^{2+}	107	121	Fe^{2+}	83	106
Mg^{2+}	120	124	Ca^{2+}	134	133
Cd^{2+}	3	1	Ni^{2+}	14	12
Cu^{2+}	44	4	Cs^+	82	79
Zn^{2+}	7	3			

Remarkably, Ker02562 was stable in the extreme alkaline environments up to pH 13.0, which was much better than most of other reported keratinases (Table 5). Most of the keratinases, particularly those from *Streptomyces* sp. and *Bacillus* sp., are described to be serine or metalloproteases (Brandelli, 2008). In this study, the enzymatic activity of Ker02562 was completely inhibited by EDTA, a chelating agent that inhibited metalloprotease, and was strongly inhibited by 5 mmmol/L PMSF, a serine protease inhibitor. Consistently, Mg^{2+} and Ca^{2+} ions can significantly enhance the activity of Ker02562, further suggesting that Ker02562 was a serine metalloproteases requiring metal ions (particularly Mg^{2+} and Ca^{2+}) for its best activity and stability.

Bacillus sp. JM7 and Ker02562 can produce essential amino acids from feather powder that were deficient in feather keratin, such as histidine, lysine, methionine and tyrosine, as well as free amino acids such as cysteine, proline and alanine (Table 2). Besides, scanning electron microscopy results showed that JM7 strain could degrade native chicken feathers gradually by modifying the microstructure of feather keratin (Fig. 1c), producing more digestive feather meal for consuming animals. Therefore, *Bacillus* sp. JM7 and its keratinase Ker02562 might be useful in biodegradation and utilization of feather keratin, which may

Table 4. Effects of chemical reagents on Ker02562 activity

Chemical reagent	Relative activity/%		Chemical reagent	Relative activity/%		
	1 mmol/L	5 mmol/L		1%	3%	5%
Control	100	100	Control	100	100	100
PMSF	92	59	DMSO	91	83	74
EDTA	9	6	Isopropanol	88	75	61
			Acetonitrile	97	86	71

Table 5. Comparison of general enzymatic properties of Ker02562 and other keratinases from various microorganisms

Source	Temperature optima/°C	Temperature stability	pH optima	pH stability	Reference
Bacterial					
Bacillus sp. JM7	40–50	50°C (70%, 1 h)	7.0–9.0	5.0–13.0	this study
Bacillus sp. P7	55	50°C (40%, 1 h)	9.0	–	Corrêa et al. (2010)
Bacillus licheniformis FK 14	60	50–60°C	8.5	5.0–7.0	Farag and Hassan (2004)
Bacillus subtilis MTCC (9102)	40	–	6.0	–	Balaji et al. (2008)
Brevibacillus brevis US575	40	<60°C	8.0	5.0–10.0	Jaouadi et al. (2013)
Actinomadura keratinilytica strain Cpt29	70	60°C	10.0	3.0–10.0	Habbeche et al. (2014)
Microbacterium sp. kr10	50	60°C	7.5	–	Thys and Brandelli (2006)
Stenotrophomonas maltophilia	40	50°C (60%, 1 h)	7.8	6.0–8.5	Cao et al. (2009)
Thermoanaerobacter sp. 1004-09	60	90°C	9.3	5.0–10.5	Kublanov et al. (2009)
Fungus					
Streptomyces gulbargensis DAS 131	45	60°C	9.0	7.0–9.0	Syed et al. (2009)
Streptomyces aureofaciens K13	75	<65°C	12.0	7.0–12.0	Gong et al. (2015)
Doratomyces microsporus	50	–	8.0–9.0	–	Gradišar et al. (2000)
Aspergillus oryzae	50	50°C (90%, 1 h)	8.0	5.0–11.0	Suntornsuk et al. (2005)
Aspergillus fumigatus TKF1	45–50	50–70°C	6.0	5.0–7.0	Paul et al. (2014)

overcome the limitations of hydrothermal process conventionally used for feather conversion.

In the detergent industry, the enzymes for detergent additive in general are alkaline, and are thermostable at medium-high temperatures, because the pH of the laundry detergent is in alkaline range (pH 9.0–11.0) and the laundry temperatures are usually 40–60°C (Aehle, 2006; Zhou et al., 2015). Thus Ker02562 might be significant in detergent industry due to its ability to operate in broad temperature and pH ranges in addition to its stability in extreme alkaline environments and medium-high temperatures (40–60°C).

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