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ORIGINAL ARTICLE

Identification and characterization of feather-degrading bacteria from keratin-rich wastes

Younes Ghasemi · Maryam Shahbazi · Sara Rasoul-Amini · Mohammad Kargar · Azam Safari · Aboozar Kazemi · Nima Montazeri-Najafabady

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Abstract In this study we aimed to isolate and identify bacteria showing high keratinolytic activity from different sources of decomposed poultry waste and soil in Shiraz, Iran. Initial screening identified 30 bacteria showing high proteolytic activity, which were selected for transfer to basal feather medium using feathers as the sole source of carbon, nitrogen and sulfur. The keratinolytic activity of the 13 isolates growing well on this medium was assayed using keratin azure as substrate. All isolates were identified based on 16S rDNA as a molecular marker. Of these, *Bacillus* sp. MKR5 exhibited the highest keratinolytic activity (225 U/ml), and was selected for further characterization. MKR5 demonstrated growth over a wide range of temperature (20–50°C). Complete feather degradation was achieved within 24 h at 40°C. This strain produced a thermostable keratinase with optimum activity at 70°C and pH 8.0. Enzyme activity was increased significantly by using 2-mercaptoethanol as a reducing agent. The keratinase was activated substantially in the presence of Co^{2+}, Mg^{2+}, TritonX-100, Tween-80 and EDTA, whereas SDS had a negative effect on enzyme activity. The thermostability of this enzyme makes it feasible to take advantage of this bacterium in biotechnological processes.

Y. Ghasemi (✉) · M. Shahbazi · S. Rasoul-Amini · A. Safari · A. Kazemi · N. Montazeri-Najafabady
Department of Pharmaceutical Biotechnology and Pharmaceutical Sciences Research Center, School of Pharmacy, Shiraz University of Medical Sciences,
PO Box 71345-1583, Shiraz, Iran
e-mail: ghasemiy@sums.ac.ir

M. Shahbazi · M. Kargar
Department of Microbiology, Jahrom Branch,
Islamic Azad University,
Jahrom, Iran

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Introduction

Keratin belongs to a family of fibrous, insoluble structural polypeptides, and constitutes the major component of the epidermis and its appendages such as hair, nails, feathers, wool and horns. According to secondary structure, keratins are grouped into α-keratin or β-keratin. On the basis of sulfur content, keratins are classified into hard (feather, hair, hoof and nail) and soft (skin and callus) keratin (Gupta and Ramnani 2006). The high degree of cross-linking of disulfide bonds and several hydrophobic interactions confer structural rigidity and mechanical stability of keratinous materials and make them resistant to proteolytic enzymes such as trypsin, pepsin or papain (Onifade et al. 1998; Brandelli 2008).

Despite this recalcitrant structure, keratins are the targets of a particular class of proteolytic enzymes named keratinases, which have been associated to the subtilisin family of alkaline serine- or metallo-proteases. Although the sophisticated mechanistic details of keratinolysis are still debated, it is clear that there are two main steps during keratin degradation: sulfitolysis or reduction of disulfide bonds, and proteolysis (Gupta and Ramnani 2006). Keratinases are predominantly extracellular, inducible enzymes (Cheng et al. 1995; Bernal et al. 2003), produced by a large number of microorganisms that grow on keratinous substrates including feathers, wool, hair and nail (Gupta and Ramnani 2006). There have been many reports of the identification of keratinolytic bacteria , mostly belonging to *Bacillus* sp. (Williams et al. 1990; Lin et al. 1999; Yamamura et al. 2002; Zerdani et al. 2004;

Korkmaz et al. 2004; Joshi et al. 2007; Cai et al. 2008; Xu et al. 2009) and other species, including *Chryseobacterium* sp. (Brandelli and Riffel 2005), *Nocardiopsis* sp. (Mitsuiki et al. 2004), *Vibrio* sp. (Sangali and Brandelli 2000), *Streptomyces* sp. (Tatineni et al. 2008; Dastager et al. 2009; Kanosh et al. 2009), *Pseudomonas* sp. (Riffel and Brandelli 2006; Tork et al. 2010) and *Lysobacter* sp. (Allpress et al. 2002). As the most plentiful insoluble keratinous waste material, poultry feathers agglomerate annually after poultry processing and represent a potent environmental pollutant (Onifade et al. 1998). Currently, they are converted to nutritional animal feedstuff (feather meal) through hydrothermal or chemical treatment (Onifade et al. 1998; Tatineni et al. 2008; Delvasigamani and Alagappan 2008), which results in the loss of essential amino acids such as methionine, lysine, histidine and tryptophan (Baker et al. 1981). The use of microbial keratinases represents a potentially improved alternative technology for recycling keratinous byproducts, leading to nutritional upgrading, as well as cost-effective and globally environment-consistent prospects (Onifade et al. 1998). The potential applications of microbial keratinases extend from bioprocessing of agroindustrial wastes, to the leather and cosmetic industry, enhanced drug delivery and some challenging areas like degradation of prion proteins, which are protease resistant and insoluble in nondenaturing detergents (Mitsuiki et al. 2006; Brandelli 2008). These various applications of keratinases have led to these enzymes being called “modern proteases“ (Gupta and Ramnani 2006).

In this report, we describe the isolation and identification of keratinolytic bacteria from local poultry wastes using 16S rDNA gene sequencing, followed by characterization of the keratinase from *Bacillus* sp. MKR5, which exhibited the highest keratinolytic activity (225 U/ml) over a wide thermal and pH range. MKR5 keratinase was able to degrade chicken feathers completely within 24 h.

Materials and methods

Isolation and screening of keratinolytic bacteria

Poultry wastes or soil samples from different poultry farms in Shiraz, Iran, were collected and serially diluted. For primary screening, all dilutions were streaked on skim-milk agar containing: 2% (w/v) skim-milk powder, 0.1% (w/v) NaCl, 1% (w/v) tryptone and 1% (w/v) agar. After 24 h of incubation at 37°C and several subculturing steps, the colonies producing the largest clearing zones on this medium, indicating the production of proteolytic enzymes, were selected for transfer to basal feather agar plates containing the following components: 0.05% (w/v) NaCl, 0.07% (w/v) KH_2PO_4, 0.14% (w/v) K_2HPO_4, 0.01% (w/v) $MgSO_4 \cdot 7\ H_2O$, 1% (w/v) agar and 1% (w/v) feather powder as the only source of carbon and nitrogen (Cai et al. 2008); the pH was adjusted to 8.0–9.0. The plates were incubated at 37°C for up to 5 days. Among 30 active isolates, 13 colonies that grew well on this medium were selected to be subcultured at frequent intervals until well-adapted and purified colonies were obtained. Initial morphological identification of isolates was done by Gram staining. These colonies were maintained subsequently on Luria-Bertani (LB) medium [1% (w/v) NaCl, 1% (w/v) water tryptone, 0.5% (w/v) yeast extract, 10% (w/v) agar] at 4°C for further work. All purified isolates were preserved at −70°C in 25% glycerol solution for long-term storage.

Keratinase production

The 13 selected colonies were incubated in LB broth for up to 20 h. As inocula, 5% (v/v) bacterial suspension from each 10^6 CFU/ml culture were transferred to 500 ml Erlenmeyer flasks containing 100 ml basal feather broth (washed and sterilized whole feathers were used instead of feather powder) and incubated at 37°C with constant agitation at 180 rpm for 5 days. Culture supernatants obtained after centrifugation at 10,000×*g* for 10 min were used as crude enzyme for assaying keratinolytic activity. To obtain the intracellular fraction of the enzyme, the cell pellet was washed three times with Tris-HCl buffer (50 mmol/l pH 8.0). After centrifugation at 10,000×*g* for 10 min, the pellet was resuspended in the same buffer and lysed in an ultra-sonic processor at 15,000 Hz for 2 min. The cell-free supernatant was used for the intracellular enzyme assay.

Assay of keratinase activity

Keratinase activity was assayed using Keratin azure (Sigma-Aldrich, Steinheim, Germany) as substrate according to the method described by Cai et al. (2008), modified slightly as follows. In brief, 4.4 mg keratin azure powder was suspended in 1 ml 50 mmol Tris buffer pH 8.0. A mixture of 1 ml diluted crude enzyme in the same buffer and 1 ml keratin azure suspension was incubated at 70°C and 150 rpm for 45 min. The reaction was then stopped by adding 2 ml 0.4 mol/l trichloroacetic acid (TCA). After centrifugating at 10,000 *g* for 10 min, the supernatant was removed and assessed photometrically in terms of releasing Azo dye at 595 nm (Eppendorf Biophotometer plus, Eppendorf, Germany). A 1 ml keratin azure suspension in the same buffer (as that of the sample) was agitated for 45 min at 70°C, and 2 ml 0.4 mol/l TCA and 1 ml enzyme solution was then added as a control.

One unit (U) of keratinase activity was defined as the amount of enzyme that caused an increase in absorbance of 0.01 between the sample and its control under the same conditions.

Protein determination

The protein concentration of the isolate was determined according to Bradford (1976) using human serum albumin as a standard.

Identification of isolates

The 13 isolates, listed in Table 1, were identified by PCR using 16S rDNA as a molecular marker. The primers used were as follows: forward primer (5′ CAGCCGCGGTAATAC 3′), and reverse primer (5′ ACGGGCGGTGTGTAC 3′). PCR was run for 30 cycles using the DNA thermal cycler (BioFlux, TC-16H, Japan). The PCR products were analyzed in a 1% (w/v) agarose gel with ethidium bromide before being sent for sequencing analysis. The DNA sequences were aligned and compared using the BLAST algorithm to find homologous sequences in the GenBank database of NCBI. The data were submitted to the GenBank database. Isolate MKR5 showed the best keratinase activity, and was also studied for its morphological, cultural and biochemical characteristics (Table 2) according to *Bergey's Manual of Systemic Bacteriology.*

Phylogenetic analysis of *Bacillus* sp. MKR5

A phylogenetic study of the selected strain, *Bacillus* sp. MKR5, was performed using the ClustalW program within MEGA4 software version 14.0.0.162 (Tamura et al. 2007). The branching pattern was designed based on the neighbor-joining method.

Effect of pH and temperature on keratinase production by *Bacillus* MKR5

To investigate the effects of temperature on enzyme production, basal feather broth flasks (pH 8.0) were incubated separately at 25, 30, 37, 40 and 45°C for 48 h under the same conditions. The basal feather medium was also prepared in the pH ranges from 5.0 to 11.0.

Effects of pH and temperature on crude enzyme of *Bacillus* MKR5

To study the optimum pH for keratinase activity, the crude enzyme was assayed in the pH range from 5.0 to 11.0 using the following buffers (50 Mm): citrophosphate (pH 5.0–7.0), Tris-HCl (pH 8.0) and carbonate-bicarbonate (pH 9.0–11.0) and keratin azure as substrate. To determine pH stability, the crude enzyme was pre-incubated in those buffers with different pH values (5.0–11.0) at 37°C for 4 h. Residual activity was then assayed (Tatineni et al. 2008).

The optimum temperature for enzyme activity was also studied. The crude enzyme was assayed at different temperatures between 37°C and 80°C. To determine thermal stability, the crude enzyme solution in Tris-HCl buffer (50 mmol/l, pH 8.0) was pre-incubated at 30–100°C in increments of 10°C for 45 min before being tested for residual keratinase activity.

Table 1 Bacterial strain, time required for complete feather degradation, total keratinase activity of the isolates using keratin azure as substrate, GenBank database accession numbers and the length of base pairs of the DNA published sequences at NCBI

Bacterial strain	Time required for complete feather degradation (h)	Maximum keratinase activity (U/ml)[a]	Accession number	Length (base pairs)
Bacillus sp. MKR1	48	108	HQ141583	420
Bacillus sp. MKR2	48	138	HQ141584	777
Bacillus sp. MKR3	96	53	_	_
Bacillus sp. MKR4	90	82	HQ149628	454
Bacillus sp. MKR5	30	225	HQ141585	816
Bacillus sp. MKR6	120	36	_	_
Bacillus sp. MKR7	100	104	HQ141588	551
Bacillus sp. MKR8	36	183	HQ141587	609
Bacillus sp. MKR9	72	94	HQ149629	785
Bacillus sp. MKR10	48	75	HQ149630	818
Bacillus sp. MKR11	48	121	HQ149631	784
Enterobacter sp. MKR12	72	103	HQ149632	585
Bacillus sp. MKR13	48	125	HQ157239	821

[a] Mean of three independent determinations

Table 2 Morphological, cultural and physiological characteristics of *Bacillus* sp. MKR5

Morphological characteristics	
Shape	Rod in single
Size	0.5-1×2 μm
Gram stain	Positive
Spore	Spore forming
Cultural characteristics	
Feather agar colonies	Creamy white color, circular, rough, H_2S after 4 days
LB agar colonies	Creamy yellowish color, mucoid, convex
Physiological characteristics	
Catalase	Positive
Oxidase	Negative
V-P test	Positive
Methyl red test	Negative
Dnase	Negative
Nitrate reduction	Positive
Citrate	Negative
Urease activity	Negative
Starch hydrolysis	Positive
Motility	Negative
Carbohydrate fermentation	Acid / no gas
Acid from arabinose	Positive
Growth in 6.5% NaCl	Positive

Effects of chemicals and metal ions on crude enzyme activity

Some chemicals, such as 2-mercaptoethanol, Isopropanol, Triton X-100, Tween 80, and SDS, and some metal ions at the different working concentrations listed in Tables 3 and 4 were added to the enzyme solution and pre-incubated for 15 min at room temperature before assaying the solution for keratinase activity.

Table 3 Effects of selected chemicals on the keratinolytic activity of *Bacillus* sp. MKR5

Chemical	Concentration	Keratinase activity (%)[a]
Control	_	100
EDTA	10 mM	150
SDS	0.1 % (w/v)	17
SDS	0.5 % (w/v)	23
2-Mercaptoethanol	0.1 % (v/v)	131
2-Mercaptoethanol	0.5 % (v/v)	187
Triton X-100	0.5 % (v/v)	165
Isopropanol	5 % (v/v)	97
Tween 80	0.1 % (v/v)	145

[a] Mean of three independent determinations

Results and discussion

Isolation and screening of keratinolytic bacteria

As the most frequent voluminous keratin wastes in nature, poultry feathers were chosen to screen keratinolytic bacteria in this study. In the preliminary screening, 30 isolates showing proteolytic activity by producing clear zones on skim-milk agar plates were selected for transfer to basal feather agar. Among these 30 isolates with proteolytic activity, 7 did not grow on basal feather agar, indicating that they did not possess keratinolytic activity. However, 13 colonies growing well on this medium were chosen for further studies. Initial morphological identification showed that 12 isolates were Gram-positive, spore-forming bacilli and 1 was Gram-negative and rod-shaped. Since there are no significant reports of isolation and identification of keratinolytic bacteria in Iran, these local isolates manifesting high keratinase activity could be of great interest to various industrial processes.

Keratinase production and enzyme assay

All 13 strains were incubated on whole feather broth at 37°C for up to 5 days and crude enzyme activity was assessed using keratin azure as substrate (Table 1). Some rapid-growing species including *Bacillus* sp. MKR1, *Bacillus* sp. MKR5, *Bacillus* sp. MKR8, *Bacillus* sp. MKR10 and *Bacillus* sp. MKR11 showed high keratinolytic activity (U/ml) and degraded whole feather keratin completely in 48 h (Table 1). Decreased in enzyme activity

Table 4 Effect of metal ions on keratinase activity of *Bacillus* sp. MKR5

Ion	Concentration	Keratinase activity (%)[a]
Control	_	100
Ca^{2+}	10 mM	83
Ca^{2+}	5 mM	90
Cu^{2+}	10 mM	105
Mg^{2+}	10 mM	132
Co^{2+}	10 mM	170
NH_4^+	10 mM	220

[a] Mean of three independent determinations

Fig. 1 Feather degradation by *Bacillus* sp. MKR5 after 24 h at 37°C (*left flask*) and autoclaved inoculum as control (*right flask*)

was observed in all these five isolates after 72 h of incubation, probably due to autolysis or product negative feedback. *Bacillus* sp. MKR5 showed the highest value of keratinolytic activity (225 U/ml) in its culture supernatant, and required the least time for complete feather degradation (less than 24 h of incubation) at 40°C and pH 8.0 (Fig. 1). Previous work has shown maximum keratinase production was achieved after 48 h of incubation at an initial pH 8.0 at 40°C during late exponential phase (Williams et al. 1990). Compared to most other isolated *Bacillus* strains (Xu et al. 2009; Zhang et al. 2009), in the case of MKR5, complete feather degradation was observed in much shorter time, which is a noteworthy criterion. Although microbial keratinases are predominantly extracellular (Cheng et al. 1995) *Bacillus* sp. MKR5 demonstrated both extracellular and intracellular keratinase activity (Fig. 2). The keratinase activity of the cell-associated lysate increased to a maximum value (70 U/ml) in 48 h and decreased after 96 h of incubation. The intracellular fraction of the enzyme presumably contributes to disulfide bonds reduction (sulfitolysis), which may support complete keratin degradation (Ramnani et al. 2005).

Protein concentration of the crude enzyme reached a maximum level at 48 h (Fig. 3), coinciding with keratinase production at the end of the exponential phase.

Identification of isolates

The PCR sequence results of the 13 isolates confirmed the primary morphological identification. The edited sequences of 12 isolates (*Bacillus* sp. MKR1–*Bacillus* sp. MKR13) showed high sequence homology to the other *Bacillus* sp., whereas 1 sequence (*Enterobacter* sp. MKR12) showed more than 99% similarity to *Enterobacter* sp. The DNA sequences of 11 species were submitted to the GenBank database and published in NCBI; the lengths of the 16S rDNA region of the species and their specific accession numbers are listed in Table 1. The feather-degrading bacterium, *Bacillus* sp. MKR5, which showed the highest enzyme activity, was chosen for further characterization.

The cultural, morphological and physiological characteristics of strain MKR5 are summarized in Table 2. Briefly, the isolate *Bacillus* sp. MKR5 proved to be Gram-positive straight rod-shaped cells with a central or subterminal oval endospore per cell. It formed opaque creamy circular colonies on feather-agar plates whereas the colonies were irregular in shape and mucoid on LB agar plates. Although most *Bacillus* species are typically mesophilic or thermotolerant, this rapidly growing strain was able to grow over a wide thermal range (25–50°C) and demonstrated facultative growth at thermophilic temperatures (40–50°C). The ability to grow

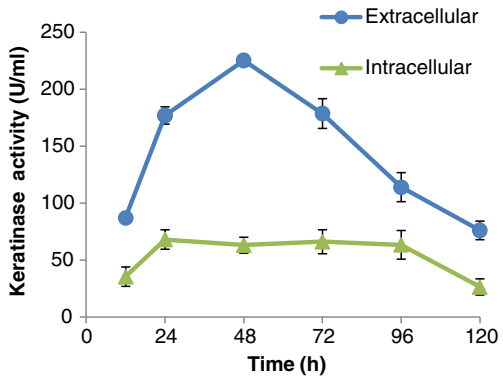

Fig. 2 Effects of cultivation time (h) on the activity of extracellular (*circles*) and intracellular fractions (*triangles*) of the keratinase from *Bacillus* sp. MKR5. The enzyme assays were performed at 70°C using keratin azure as substrate. Each point represents the mean of three independent experiments. *Bars* Standard error of the means

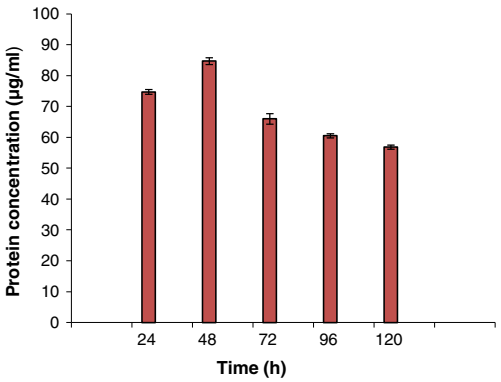

Fig. 3 Concentration of soluble protein in crude enzyme of *Bacillus* sp. MKR5. Each point represents the mean of three independent experiments. *Bars* Standard error of the means

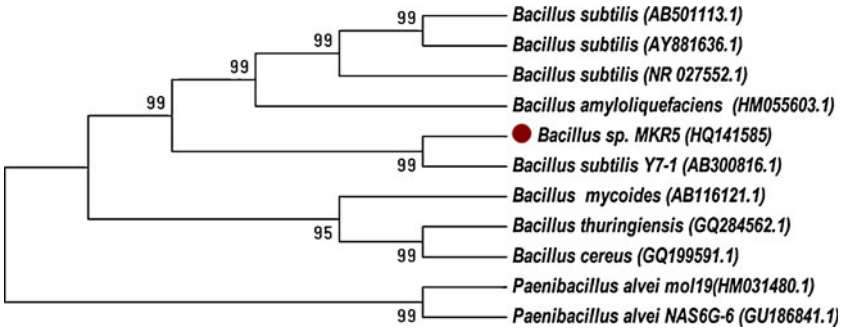

Fig. 4 Phylogenetic position of *Bacillus* sp. MKR5 based on 16S rDNA sequence within the genus *Bacillus*. The sequences were aligned using the Clustal W program and MEGA4 software. The branching pattern was based on the neighbor-joining method

in 6.5% NaCl categorizes this strain as a halotolerant microorganism (Tiquia et al. 2007).

Phylogenetic analysis of the selected isolate *Bacillus* sp. MKR5

The taxonomic position was confirmed by phylogenetic analysis based on 16S rDNA sequences (Fig. 4). A total of 816 nucleotides of partial sequences of *Bacillus* sp. MKR5 (HQ141585) revealed 99–100% sequence identity to *Bacillus subtilis* Y7-1 with sequence accession no. AB300816.1. Due to the high sequence homology of the partial sequences of this bacterium with other *Bacillus* using the BLASTn algorithm, this strain was submitted to the GenBank database as a *Bacillus* species.

Effect of pH and temperature on keratinase production by *Bacillus* MKR5

Maximum keratinase production was achieved at 40°C and pH 8.0 after 48 h. Korkmaz et al. (2004) indicated that optimum keratinase production for *Bacillus licheniformis* HK-1 was found with at initial pH of 8.0, which is similar to our finding. The optimal temperature for keratinase production by *B. subtilis*, *B. cereus* and *B. pumilis* was 40°C, 30°C and 40°C, respectively (Kim et al. 2001).

Effects of pH and temperature on crude keratinase enzyme activity of *Bacillus* MKR5

The keratinase from *Bacillus* sp. MKR5 was found to be active at neutral and alkaline ranges of pH (5.0–10.0), but exhibited optimum activity and enzyme production at pH 8.0 (Fig. 5). Stability of keratinase was observed over the pH range 7.0–9.0 (Fig. 5). Similar results have been reported from other *Bacillus* sp. keratinases. For example, the optimum activity of keratinase from *B. subtilis* KD-N2 was at pH 8.5 (Cai et al. 2008), and that of *B. licheniformis* K-19 was at pH 7.5–8.0 (Xu et al. 2009).

Due to its high keratinolytic activity, the pH increased significantly (from 8.0 to 10.0) during cultivation and alkalinized the medium, which is a result of peptide deamination reactions and ammonium production (Riffel et al. 2003).

The enzyme was found to be stable over a wide range of temperature (40–70°C). Above 100°C, enzyme activity

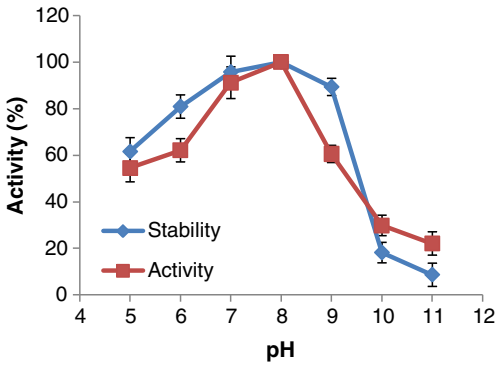

Fig. 5 Effect of pH on keratinase activity from *Bacillus* sp. MKR5. Activity (*squares*) was assayed at the indicated pH values: citrophosphate (pH 5.0–7.0); Tris-HCl (pH 8.0) and carbonate (pH 9.0–11.0) buffers. Effect of pH on the enzyme stability was also measured (*diamonds*). Each point represents the mean of three independent experiments. *Bars* Standard error of the means

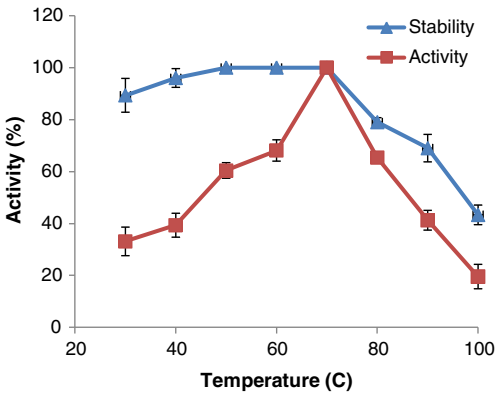

Fig. 6 Effect of temperature on keratinase activity from *Bacillus* sp. MKR5 (*squares*). Thermal stability of keratinase (*triangles*). Each point represents the mean of three independent experiments. *Bars* Standard error of the means

decreased rapidly (Fig. 6). The enzyme was also active at room temperature for 10 days without any significant change in its activity. The optimum temperature for keratinase activity of *Bacillus* sp. MKR5 was 70°C (Fig. 6), much higher than that of other *Bacillus* keratinases (50–55°C) (Lin et al. 1999; Cheng et al. 1995; Cai et al. 2008). The thermostability of this enzyme makes it a suitable candidate for biotechnological approaches.

Effect of selected chemicals on crude keratinase enzyme activity

The effects of selected chemicals and metal ions at different working concentrations on keratinase enzyme activity were summarized in Tables 3 and 4. This keratinase was strongly inhibited by SDS, which differs from other keratinases such as *B. subtilis* KD-N2 keratinase (Cai et al. 2008). NH_4^+, Mg^{2+}, Co^{2+}, and surfactants like TritonX-100 and Tween 80 increased the enzyme activity while Cu^{2+}, Ca^{2+} and isopropanol had no significant effect. Unlike most keratinases (Allpress et al. 2002; Riffel et al. 2003; Xu et al. 2009), the keratinase produced by strain MKR5, was not inhibited by chelating agents such as EDTA, similar to the keratinase from *B. subtilis* KD-N2. Thus it cannot be categorized as metalloprotease type (Rao et al. 1998) and should probably be considered as a serine alkaline protease. With the exception of Ca^{+2} and Cu^{+2}, metal ions stimulated the enzyme activity of *Bacillus* sp. MKR5. They might play an important role in stabilization of the enzyme active site, and help maintain enzyme structural conformation. The reducing agent, 2-mercaptoethanol, enhanced enzyme activity through disulfide bonds reduction to bring about complete feather degradation (Gupta and Ramnani 2006).

The ability of *Bacillus* sp. MKR5 to grow fast over wide thermal and pH ranges on an abundant and inexpensive substrate like feathers, suggests the feasible use of this strain in commercially biotechnological processes, particularly for bioconversion of feathers into protein-rich feed stuff (Bernal et al. 2003).

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