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Isolation of a feather-degrading *Bacillus subtilis* strain from the alimentary tract of grebes

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Grebes eat feathers, but the function of feathers in the alimentary tract and the digestion of feathers are poorly understood. In this article, we isolated *Bacillus subtilis,* a bacterium with strong feather-degrading ability, from the alimentary tract of grebes; we further purified a secretory keratinase with a molecular weight of 42KDa from *B. subtilis*. The optimal pH of the keratin-degrading reaction was 6.5, which exactly was the pH value in the alimentary tract of grebes. The feathers eaten by grebes can protect wall of the stomach. The feather net in the third chamber of the stomach could filter food and prevent petrous food such as bones from entering the alimentary track. Importantly, feathers could be digested by feather-degrading bacterium to supply the specific amino acids (cysteine and cystine) after fulfilling their protection function. It was suggested that the digestion of feathers in the alimentary tract of grebes was accomplished by the keratin-degrading bacteria.

Key words: Grebes, feather-eating behavior, feather-degrading bacterium, keratinase.

INTRODUCTION

The unique feather-eating behavior in grebes has already been reported (Hanzák, 1952; Simmons, 1956; Storer, 1969; Piersma and Van Eerden, 1989), but there are still some different explanations for this behavior. It was recorded in the Handbook of the Birds of the World (Del Hoyo et al., 1992) that grebes are used to eating their own feathers and picking up subaqueous feathers. These feathers are accumulated in the muscular stomach, decomposed into the spongy green substance and form a pellet. Some of the feathers are digested definitely. However, the digestive mechanism was not mentioned. In China the feather-eating behavior in grebes was also recorded, but no specific report or research was made (Fu, 1987; Zheng, 1995). We found more than 50% contents of grebes' pellet were feathers through the dissection of the alimentary tract of grebes (Meng et al., 2002). The previous report (Del Hoyo et al., 1992) said that some of the feathers in grebe's muscular stomach were digested and the others were thrown up. But the field observations showed that all of the feathers eaten by grebes were not thrown up (Zhao, 2003).

The component of feathers is mostly β-keratin (80-90% on dry mass basis) (Fisher et al., 1981; Lee et al., 2002; Ghosh et al., 2008). In its native state, the feather keratin is insoluble and not degradable by commonly known proteolytic enzymes such as trypsin, pepsin, and papain not only because of their tight secondary structure but also because the peptide chains are held together by disulfide linkages (Williams et al., 1990; Kim et al., 2001; Schrooyen et al., 2001). Nonetheless, feathers do not accumulate in nature and can be degraded efficiently by a myriad of microorganisms due to the action of

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keratinolytic proteases—keratinases (Onifade et al., 1998). These enzymes have the ability to degradenative keratin into smaller peptide entities and amino acids that can subsequently be absorbed by cells. At present, the keratinolytic activity has been reported for *Bacillus* species (Williams et al., 1990; Kim et al., 2001; Hu et al., 1995; Yao and Yan, 2001; Suntornsuk and Suntornsuk, 2003; Manczinger et al., 2003; Suntornsuk et al., 2005; Kojima et al., 2006; Giongo et al., 2007; Nilegaonkar et al., 2007; Cai et al., 2008b; Balaji et al., 2008; Park and Son, 2009; Mazotto et al., 2011; Jeong et al., 2010), *Fervidobacterium* sp. (Riffel and Brandelli, 2002; Nam et al., 2002), *Thermoanaerobacter* sp. (Riessen and Antranikian, 2001), *Xanthomonas* sp. (De Toni et al., 2002), *Stenotrophomona smaltophilia* DHHJ (Wang et al., 2007), *Vibrio* sp. (Sangali and Brandelli, 2000), *Virgibacillus pantothenticus* (Gupta et al., 2008), *Microbacterium* sp. (Thys et al., 2004) and *Candida parapsilosis* (Vermelho et al., 2010), as well as fungi (Friedrich et al., 2005; Tan et al., 2005; Marcondes et al., 2008), *Actinomycetes* (Chitte et al., 1999; Gousterova et al., 2005; De Azeredo et al., 2006; Mabrouk, 2008), etc.

Comprehensive reviews about keratinases and their potential applications have been published (Onifade et al., 1998; Hu et al., 1995; Gupta and Ramnani, 2006; Shih and Wang, 2006; Brandelli, 2008). The keratinases from *Bacillus* sp., particularly *B. licheniformis* and *B. subtilis* have been studied extensively due to their effectiveness in terms of feather degradation (Manczinger et al., 2003; Thys et al., 2004). Some isolates have been described to degrade feathers completely in culture medium, such as *B. licheniformis* PWD-1 (Williams et al., 1990), *B. pseudofirmus* FA30-01 (Kojima et al., 2006), *B. megaterium* F7-1 (Park and Son, 2009), *B. subtilis* 1271 (Mazotto et al., 2011) and *B. subtilis* S8 (Jeong et al., 2010). In particular, Hu (1995) isolated a *Bacillus subtilis* with strong feather-degrading ability from the alimentary tract (strong acidic, pH 0.7~3.0) of cormorant (*Phalacrocorox carbo sinensis*), and made enzymolysis trials of feathers successfully. The alimentary tract of Grebes is weak acid (pH 5.4~6.5) and it is more suitable for bacterial survival. Therefore, we made selective enrichment culture for the microbial pool of the alimentary tract of grebes under the stimulant avian temperature and pH condition, in order to identify a keratin-degrading bacterium and study the digestive mechanism of feathers.

MATERIALS AND METHODS

Experimental animals

Tachybaptus ruficollis, *Podiceps cristatus* and *Podiceps rigricollis* were collected from Wuliangsuhai Lake of Inner Mongolia Autonomous Region in China.

Cultivation media

The enrichment medium used for enriching bacteria in the alimentary tract of grebes contained 3% (w/v) beef extract; 8% (w/v) peptone; 0.5% (w/v) NaCl, and 10% (w/v) whole feathers, at pH 6~7. The selective medium containing 0.5% (w/v) NaCl and 1% (w/v) whole feather which was the sole sources of carbon and nitrogen was used for selecting feather-degrading bacteria. The feather meal agar medium containing 0.5% (w/v) NaCl, 1% (w/v) feather meal and 2% (w/v) agar was used for isolating feather-degrading bacteria in plates. The whole feather liquid medium was used for primary screening of the strain with strong feather-degrading ability, and the feather meal (1-2%, w/v) liquid medium was used for duplicate screening of the strain with strong feather-degrading ability. The fermentation medium used for the cultivation of the feather-degrading bacterium contained the following components (w/v): 0.5% NaCl, 0.3% K_2HPO_4, 0.4% KH_2PO_4, 0.1% $MgSO_4$ and 10% feather meal, pH 6~7.

Sample and substrate preparation

The grebe alimentary tracts were dissected immediately after collection and their liquid contents were diluted in sterile water and adjusted to 10% (w/v) specimen solution.

Feathers were washed extensively with water and detergent, and dried in a ventilated oven at 40°C for 72 h. To prepare the feather meal, feathers were milled in a ball mill and passed through a small-mesh grid to remove coarse particles.

Enrichment and isolation of feather-degrading bacteria

We inoculated 0.5 ml of 10% (w/v) specimen solution into the enrichment medium and cultured it at 42°C for 7~12 days under static conditions. A control abiotic sample containing only feathers without added inoculum was cultivated under the same conditions. The effective bacterial culture fluid in which the feathers were broken down was inoculated at 42°C for 7~12 days in a test tube containing the selective medium. The effective bacterial culture fluid was streaked on feather meal agar plates, and incubated at 42°C for 3 days. Single colonies were isolated and screened for their ability to hydrolyse keratin in feather meal agar plates. Colonies producing clearing zones in this medium were selected and cultured at 42°C for 3~5 days in the test tube containing the whole feather liquid. The feather-degrading ability was visually inspected at 24 h intervals (Riffel and Brandelli, 2002). One milliliters culture fluid (the degradation extent of feathers > 50%) was inoculated in a 500-ml Erlenmeyer flask containing 100 ml feather meal liquid medium. The cultures were incubated at 40°C and 120 r/min for 60 h in a gyratory shaker. At periodic intervals, the cultures were centrifuged and filtered through a Xinhua filter paper, and then dried to a constant weight. The strain with strong feather-degrading ability (the degradation rate of feather meal > 50%) was selected for further experiments.

Identification of feather-degrading bacteria

Identification of the isolates was carried out based on the observed results from light microscope and transmission electron microscope as well as parts of physiologic and biochemical experiments (Dong and Cai, 2001) according to the eighth edition book of Bergey's Manual of Systematic Bacteriology (Buchanan and Gibbons, 1984).

Genomic DNA from the strain P-20 was isolated as described by Sambrook et al. (1989). The 16SrDNA gene was amplified by PCR using primers 5′-GCG TGC CTA ATA CAT GCA AG-3′ and 5′-AAG GTT ACC TCA CCG ACT TC-3′ designed from the conserved sequences of *B. subtilis* strains. BLAST algorithm was used to search for homologous sequences in GenBank. The 16S rDNA sequences were aligned using the ClustalX program (Thompson et al., 1997) and the phylogenetic tree was booted by the MEGA3 soft-

ware (Kumar et al., 2004).

Measurement of population growth

The P-20 (24-h-old) was inoculated in a 500-ml Erlenmeyer flask containing 100 ml feather meal liquid medium and incubated at 40°C and 120 r/min for 108 h in a gyratory shaker. Cell mass was measured at A_{550} against a control by visible spectrophotometer at 12 h intervals. The control was an abiotic sample containing only feather meal without added inoculum cultivated under the same conditions. Microbial growth curve was drawn according to the value of optical density.

Determination of enzyme activity

For measuring Caseinolytic activity, the crude enzyme (1 ml) was mixed with 1 ml of 2% casein in phosphate buffer (pH 7.0) and incubated for 10 min at 40°C. The reaction was terminated by adding 2 ml of 0.4 mol/l trichloroacetic acid (TCA). The reaction mixture was centrifuged and the soluble peptide in the supernatant fraction was measured with tyrosine as the reference compound (Liang et al., 2006) at 680 nm. Protein concentration was measured by the method of Lowry et al. (1951) with bovine serum albumin as standard. One unit (U/ml) of Caseinolytic activity is defined as the activity that releases 1 µg tyrosine in 1 min at 40°C.

Keratinase activity was measured using feather meal as substrate (Yao, 2001). Both feather meal and 0.2 ml of enzyme solution were incubated at 42°C (water bath) in 3.8 ml of 0.1mol/L Tris-HCl buffer (pH 7.2) for 1 h, the mixture was cooled down on ice for 10 min, the absorbance of the supernatant was determined at 280 nm against a control after removing the feather meal. The control was prepared by incubating the enzyme solution with 3.8 ml of 0.1mol/L Tris-HCl buffer (pH 7.2) without the addition of feather meal. One unit (U/ml) of keratinase activity was represented by an increase in A_{280} of 0.1 after 1 h.

Preparation of the crude concentrated keratinase solution

The feather-degrading bacterium (24-h-old) was inoculated in a 500-ml Erlenmeyer flask containing 100 ml feather meal liquid medium and incubated at 40°C and 120 r/min for 60 h in a gyratory shaker. The fermented broth was centrifuged at 5,000 r/min for 15 min to remove the precipitations. The keratinase was fractionated by salting out with $(NH_4)_2SO_4$ (Ranjitha and Kaushik, 2005). 30% $(NH_4)_2SO_4$ saturation was added into the supernatant to precipitate and remove impurity protein at 4°C. The keratinase was precipitated completely with 55% $(NH_4)_2SO_4$ saturation and collected after centrifuging at 12,000 r/min for 30 min. The pellets were dissolved in a small amount of 50 mmol/l potassium phosphate buffer (pH 7.2) and dialyzed overnight against the same buffer. The partially purified enzyme was used for further studies. Enzyme activity in the supernatant was measured when the keratinase was precipitated step by step using different percent $(NH_4)_2SO_4$ saturation.

Keratinase purification

The crude concentrated keratinase solution was added into a Shephadex G-75 (1.5 cm×90 cm) column. The equilibration and elution were carried out with 50 mmol/l potassium phosphate buffer (pH 7.2) at a flow rate of 0.3 ml/min. The fraction size was 3 ml (Suntornsuk et al., 2005). Protein Concentration of each fraction was measured at A_{280} by an ultraviolet spectrophotometer with bovine serum albumin as standard (Zhou, 2005). At the same time, the keratinase activity of each fraction was measured. The keratinase active fractions were collected according to the elution profile of keratinase and proteolytic activity, precipitated with 100% saturation of $(NH_4)_2SO_4$, dialyzed and lyophilized. The active fractions were pooled, concentrated and checked on SDS-PAGE.

Sodium dodecyl sulphate-polyacrylamide gel electrophresis (SDS-PAGE) electrophoresis

SDS-PAGE was performed on a slab gel (vertical) containing 15% (w/v) polyacrylamide. After the electrophoresis, the gels were stained with Coomassie Brilliant Blue and analyzed by gel imaging and analysis system (Alpha-220, Anlai Corp., China).

Effects of substrates on keratinase production

Casein, hair, peptone, skim milk powder and wool as well as feather were used as sole sources of carbon and nitrogen source for keratinase production. Cultivation was performed at 120 r/min and 40°C for 60 h.

Effect of pH and temperature on keratinase activity

The effect of pH on enzyme activity was determined by incubating the reaction mixture at various pH ranging from 2.0 to 11.0 using different buffer systems. The buffers used for the purpose were 50 mmol/l glycine-HCl (pH 2.0-6.0), 50 mmol/l Tris-HCl (pH 6.0-9.0), and 50 mmol/l glycine-NaOH (pH 9.0-11.0).

The effect of temperature for enzyme activity was determined by conducting the assay at various temperatures from 20 to 80°C. The thermostability of enzyme was measured after preincubation of enzyme in the same buffer for 1 h at various temperatures (20–80°C).

RESULTS

Isolation and identification of feather-degrading bacteria

After selective enrichment culture and plate screening of samples from the alimentary tracts of Grebes, 45 colonies were isolated and incubated under static conditions for 3~5 days. The culture fluid (Figure 1) in which the feathers were degraded mostly or entirely was collected for a shaker-flask duplicate screening. Seven *Bacillus* strains (the degradation rate of feather meal > 50%) were screened. Light microscope and transmission electron microscope as well as parts of physiologic and biochemical experiments show that all of the strains belong to *Bacillus*. The P-20 strain had the strongest feather-degrading ability (the degradation rate of feather meal is 78%) and was further studied. The strain was a Gram-positive, spore-forming, rod-shaped bacterium. The strain was further identified as *Bacillus subtilis* by China Center for Type Culture Collection (CCTCC, Wuhan). The 16SrDNA sequence showed high levels of sequence similarity to the *B. subtilis* strains of accession Nos. AJ276351 (99%) (Figure 2).

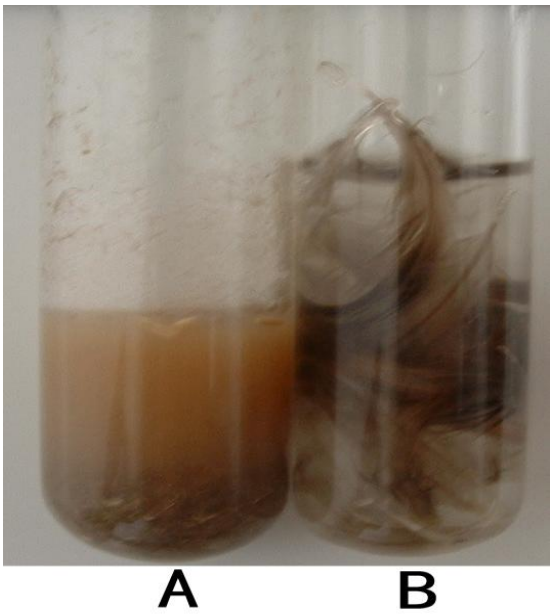

Figure 1. Screening of the strains with strong feather-degrading ability. **A.** The strains with strong feather-degrading ability were screened by feather medium. The pinnule broke off mostly or entirely and only the feather handle remained in bacterial solution after being digested by the strain with strong feather-degrading ability. **B.** The pinnule almost didn't break off and integral feathers were visible without the digestion from a feather-degrading bacterium.

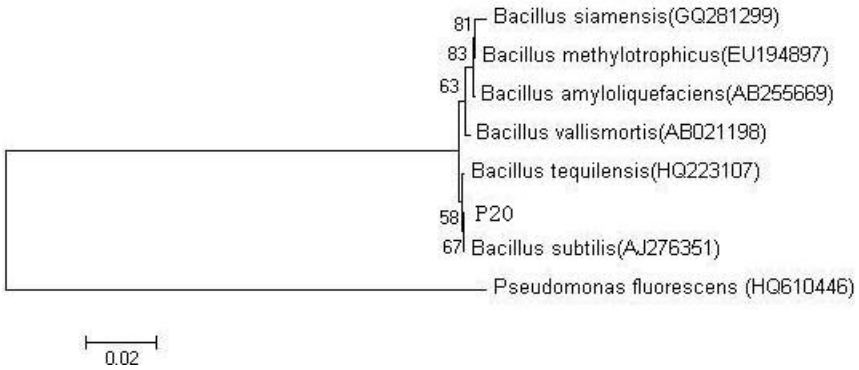

Figure 2. Phylogenetic tree based on 16S rDNA sequence of the isolate P-20. The sequences were aligned using ClustalX program and the phylogenetic tree was booted by MEGA3 software.

Establishing the extraction conditions of keratinase

The cell growth and protease production of P-20 were assayed at various fermentation time ranging from 12 to 108 h. Microbial populations enter the exponential phase when incubated more than 12 h, and enter the stationary phase when incubated more than 48 h. The enzyme activity reached maximum when the strain was fermented for 60 h, then it started to decrease (Figure 3). Therefore, the enzyme was separated at this time point. Some of the impurities were removed and no secretory keratinase of P-20 was precipitated at 30% $(NH_4)_2SO_4$ saturation. Keratinase activity (0.98U/ml) in the supernatant nearly has no change comparing with the initial keratinase activity (1.04 U/ml). However, when the saturation increased to 55%, the keratinase activity in the supernatant decreased rapidly, it was 0.09U/ml. The keratinase was precipitated completely (Figure 4).

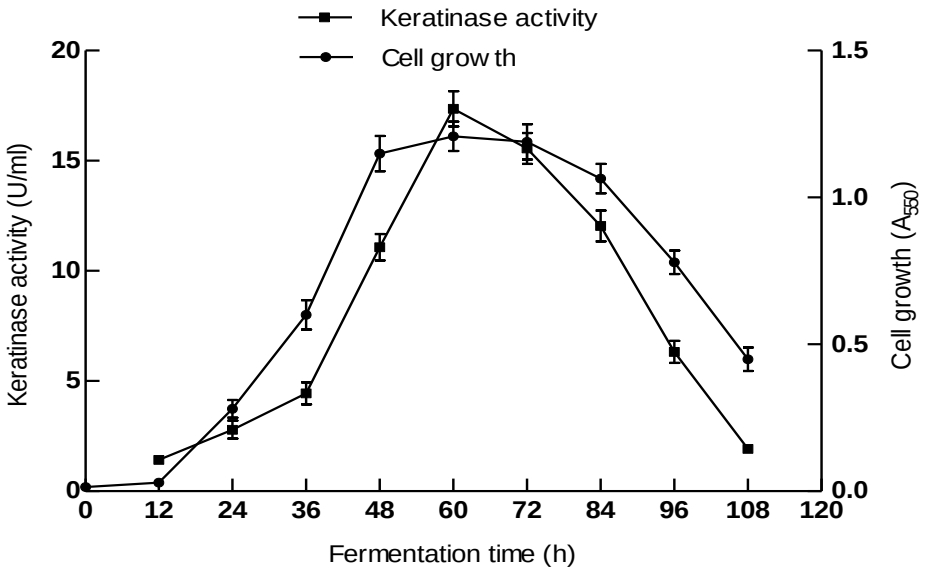

Figure 3. Growth curve and protease production of P-20. The cultivations were incubated at initial pH 6.5, 40°C and 120 r/min with 2% 24-h-old inoculum. Error bars correspond to standard deviations from triplicate replicas.

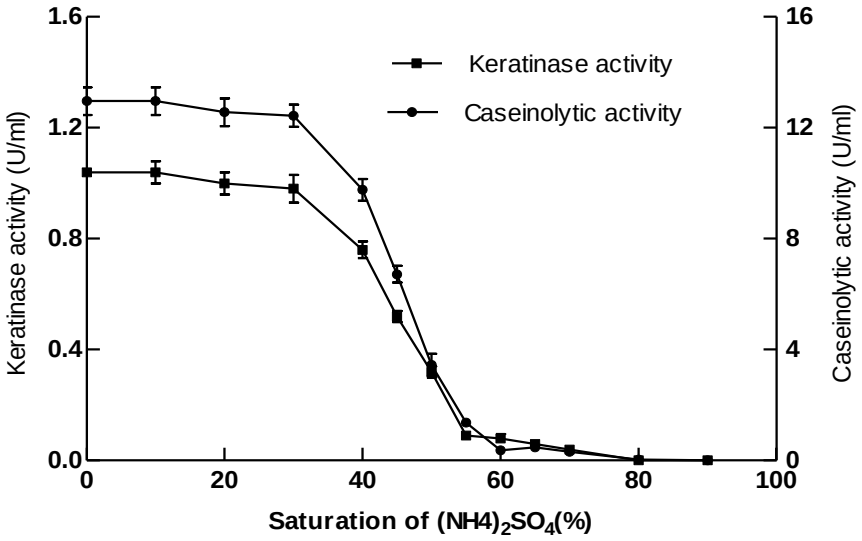

Figure 4. Salting-out curve of keratinase of P-20. The keratinase was precipitated completely at 55% saturation of $(NH_4)_2SO_4$. Error bars correspond to standard deviations from triplicate replicas.

Purification of keratinase

The desalted extract was further purified on Sephadex G-75 gel filtration chromatography. The A_{280} was measured for each fraction. Only one keratinase active peak (corresponding to fraction 45 to 74) appeared on Sephadex G-75 (1.5cm×90cm) column as determined by the keratinase activity assay (Figure 5). The purity of keratinase increased 4.51-fold by ammonium sulphate precipitation and the final yield was 38.8% at 5.46 U/mg specific activity (Table 1). Analytical SDS-PAGE (15%) showed that the purified keratinase fraction had reached electrophoresis purity and its molecular weight was determined to be around 42 KDa (Figure 6).

Effects of substrates on keratinase production

Microbial keratinases are inducible enzymes, as reported by many authors (Malviya et al., 1992). The strain P-20

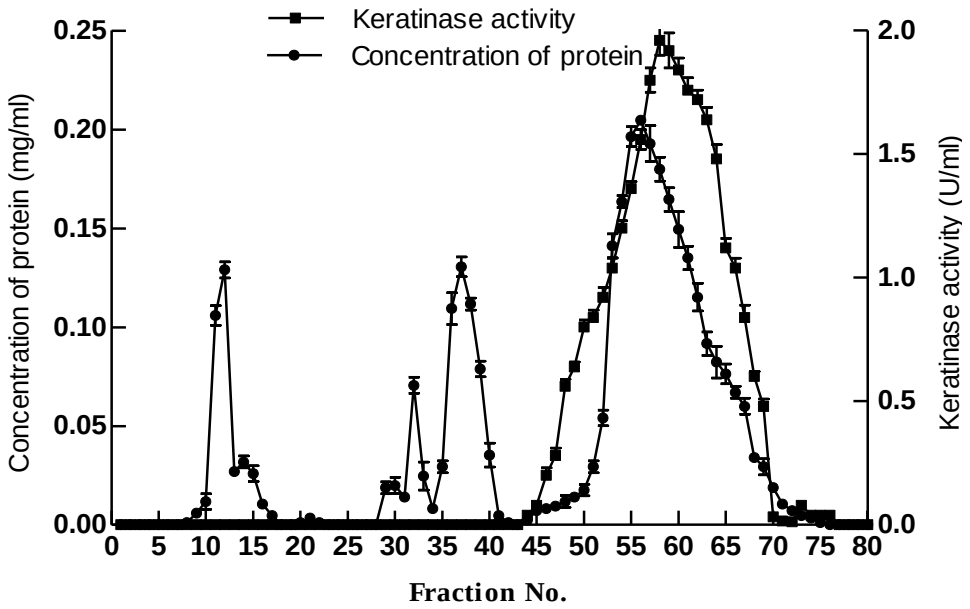

Figure 5. Elution profile of keratinase on Sephadex G-75 column and proteolytic activity in different fractions**.** The keratinase activity appeared between fraction 45 to 74. Error bars correspond to standard deviations from triplicate replicas.

Table 1. Summary of purification procedure of keratinase from *B.subtilis* P-20.

Precipitation step	Total activity (U)	Total protein (mg)	Specific activity (U/ml)	Purified (fold)	Yield (%)
Crude enzyme	1208	850	1.21	1	100
$(NH_4)_2SO_4$ precipitation (dialyzed)	399	73	5.46	4.51	38.8

produced inducible keratinase when keratin-containing materials such as feathers, hair and wool were used as sole substrates. Mostly keratinases belong to the subtilisin family of serine proteases with a cysteine protease, which have higher activity on casein (Sangali and Brandelli, 2000). In contrast, in our study it was found that the enzyme preferred keratin-based substrates rather than other soluble substrates like peptone, casein. Feather was the optimal substrate for keratinase production (Figure 7).

Effect of pH and temperature on keratinase activity

The effect of pH on enzyme activity at 42°C was determined by incubating the reaction mixture at various pH ranging from 2.0 to 11.0 using different buffer systems. As a result, the active range of pH for keratinase was between pH 6.0 and pH 9.0 whereas the optimal reaction pH of keratinase was determined to be 6.5 (Figure 8), differing from the reported keratinases from *B. subtilis* strains (Suh and Lee, 2001; Cai et al., 2008b; Balaji et al., 2008; Rai et al., 2009; Jeong et al., 2010; Mazotto et al., 2011).

High level of keratinase activity was observed in a range of 30-60°C. Optimal temperature for keratinase activity was found to be 40°C and it was 90.5% stable at this temperature for 1 h. Further increase in the temperature to 70°C reduced the relative activity to 58.7% as shown in Figure 9. Keratinolytic bacteria often exhibit optimal growth and activity at higher temperatures (Lin et al., 1999) and the reported *Bacillus subtilis* usually showed optimal keratinase production at temperatures ranging from 30 to 50°C, for example, *B*. *subtilis* S8 at 30°C (Jeong et al., 2010), *B*. *subtilis MTCC9102* at 40°C, *B*. *subtilis* 1271 at 50°C (Mazotto et al., 2011). The high thermostability allows performance of the industrial process at high temperature and minimizes the risk of microbial contamination (Balaji et al., 2008). It was suggested that the stability of protease enzyme could be due to their genetic adaptability to carry out their biological activity at a higher temperature (Whittle and Bloomfield, 1999; Kanekar et al., 2002).

DISCUSSION

The function of feathers in grebes' digestive system

The surface of food pellet in the muscular stomach of

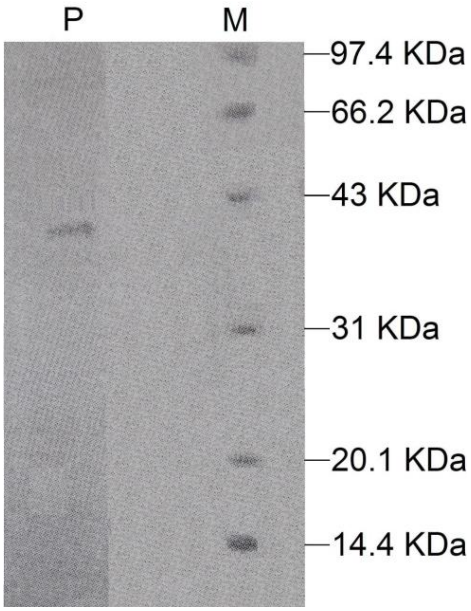

Figure 6. Purity and molecular weight of keratinase from *B.subtilis* P-20 determined by SDS-PAGE. P: Purified keratinase, M: Low molecular weight protein standard (Concentration of proteins:12 µg/µL, Component proteins: rabbit phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; rabbit actin, 43 kDa; bovine carbonic anhydrase, 31 kDa; trypsin inhibitor, 20.1kDa; lysozyme,14.4 kDa).

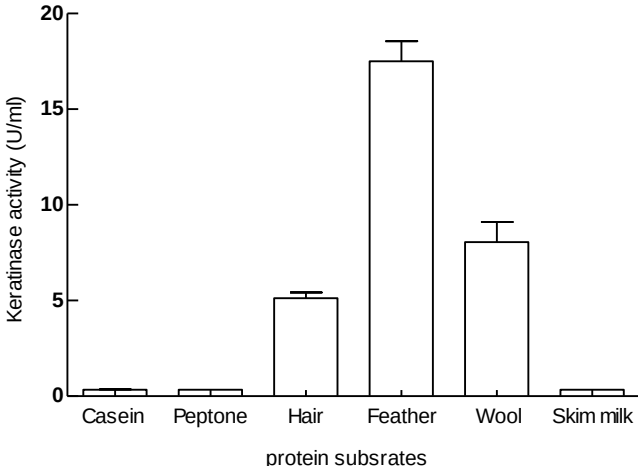

Figure 7. Effects of substrates on keratinase production. The cultivations were performed at initial pH 6.5, 40°C and 120 r/min for 60 h with 2% 24-h-old inoculum. Error bars correspond to standard deviations from triplicate replicas.

grebes was wrapped up by intact feathers, and there were even fish bones which are very hard to be digested. The feather-wrapped petrous food could not stab the stomach wall of grebes, and the helical feathers (Figure 10) in the third chamber of the stomach could filter food

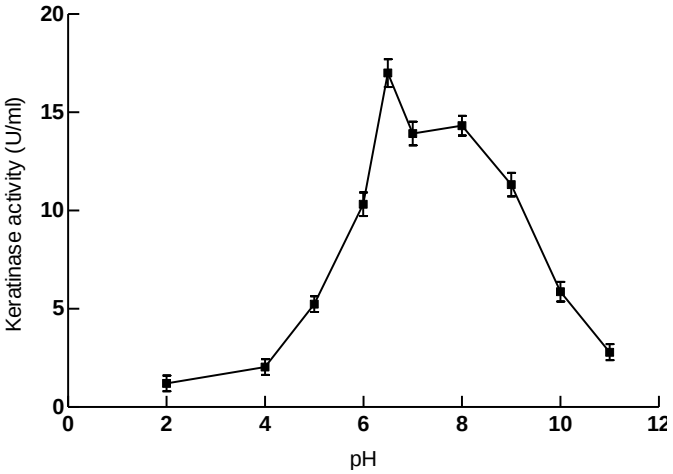

Figure 8. Effects of pH on keratinase activity at 42°C using different buffer systems. The cultivations were incubated at initial pH 6.5, 40°C and 120 r/min with 2% 24-h-old inoculum. Error bars correspond to standard deviations from triplicate replicas.

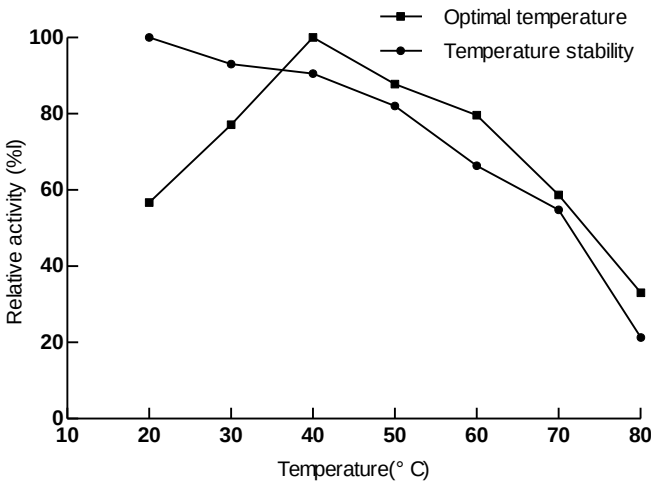

Figure 9. Optimal temperature and temperature stability of keratinase from *B. subtilis* P-20. The relative activity (%) was calculated relative to the case of reaction at which maximum activity was taken as 100%.

Figure 10. The helical feather conglomeration (from Zhao, 2003).

and prevent undigested food to enter the intestinal tract of grebes.

The shapes of feathers in grebes' muscular stomach varies (integrity, flocculence and pinnule), but no feather remnant was found in the intestinal tract of grebes and feces. It was recorded (Del Hoyo et al., 1992) that feathers eaten by grebes were digested partially. However, our observation proved they were digested completely in the stomach. Grebes grow their feathers throughout the year (Piersma, 1988), especially feathers at breast, abdomen and flank. The growth of neonatal feathers needs lots of amino acids, especially the amino acids that could be absorbed only from food instead of being synthesized, such as cystine and cysteine. Keratin is the main composition of feathers and has high contents of cystine and cysteine. Grebes take food by economical means—pecking their own feathers. Our data suggests the feathers eaten by grebes can serve as alimentation and energy sources, protect stomach wall as well. In the light of avian energetics, the autologous cycle of energy is reasonable.

The digestion mechanism of feathers

According to the degradation experiment of feathers (Figure 1), the pinnule exfoliated mostly or entirely and only the feather handle remains in the bacterial culture solution after being digested by the strain with strong feather-degrading ability. On the contrary, the pinnule almost did not exfoliate and integrity feathers were visible without the digestion of the feather-degrading bacterium. Keratinases with molecular masses ranging from 15 to 240 kDa have been reported (Gupta and Ramnani, 2006; Mazotto et al., 2011; Brandelli, 2010). From the literature, a purified keratinase from the *B. subtilis* strain KS-1 was described as a single polypeptide of 25.4 kDa (Suh and Lee, 2001). Moreover, peptidases and keratinases from the *B. subtilis* strain 1271 and *B. licheniformis* 1269 were characterized to be in the molecular range of 15~140 kDa (Mazotto et al., 2011). In this study, a secretory keratinase was purified with the molecular weight of 42KDa. The optimal pH was determined to be 6.5. The degradation of feathers most probably due to the effects of keratin-degrading bacteria.

The feather keratin is an abundant native protein resource, especially, for necessary amino acid, such as cystine and cysteine (Zhou, 1996). However, the feather keratin is a rigid protein with a very strong protease resistance and cannot be degraded by proteases from animals. One of the possible mechanisms of degradation of keratin is reduction of disulfide bonds (Onifade et al., 1998). Our results suggested that *B. subtilis* P-20 had the disulfide-bond reducing ability. Many *Bacillus* species have been reported to produce keratinolytic proteases (Nilegaonkar et al., 2007; Giongo et al., 2007; Sousa et al., 2007; Son et al., 2008). These isolates can degrade feathers and produced soluble proteins and amino acids (Grazziotin et al., 2006; Ghosh et al., 2008; Mabrouk, 2008; Mazotto et al., 2011; Jeong et al., 2010). From literature, 19 amino acids including all essential amino acids were detected in cell-free culture supernatant of *Bacillus subtilis* S8. Proline, histidine and phenylalanine were the major amino acids released in the culture medium, followed by cysteine (Jeong et al., 2010). Moreover, the nutritional upgrade of feather meal and use of microbial feather lysate in feed trials showed that the use of keratinase increased significantly the amino acid digestibility of feather keratin (Odetallah et al., 2003; Grazziotin et al., 2006). The optimal reaction pH of purified keratinase (pH 6.5) matches the weak acidic condition of the alimentary tract of grebes. Our data suggest the digestion of feathers in the alimentary tract of grebes was accomplished by a keratin-degrading bacterium.

Although keratinolytic enzyme assay methods and growth media are different and it is difficult to compare our results with those in the literature, *B. subtilis* P-20 appears suitable for the bacterial degradation of avian feathers and feather meal with a potential for biotechnological processes. The desirable properties of this protease, such as stability at a broad range of pH and high temperature permit its potential applications to be exploited in feather waste utilization.

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