Production and Characterization of Keratinase from *Paracoccus* sp. WJ-98

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Abstract A bacterial strain WJ-98 found to produce active extracellular keratinase was isolated from the soil of a poultry factory. It was identified as *Paracoccus* sp. based on its 16S rRNA sequence analysis, morphological and physiological characteristics. The optimal culture conditions for the production of keratinase by *Paracoccus* sp. WJ-98 were investigated. The optimal medium composition for keratinase production was determined to be 1.0% keratin, 0.05% urea and NaCl, 0.03% K_2HPO_4, 0.04% KH_2PO_4, and 0.01% $MgCl_2 \cdot 6H_2O$. Optimal initial pH and temperature for the production of keratinase were 7.5 and 37°C, respectively. The maximum keratinase production of 90 U/mL was reached after 84 h of cultivation under the optimal culturing conditions. The keratinase from *Paracoccus* sp. WJ-98 was partially purified from a culture broth by using ammonium sulfate precipitation, ion-exchange chromatography on DEAE-cellulose, followed by gel filtration chromatography on Sephadex G-75. Optimum pH and temperature for the enzyme reaction were pH 6.8 and 50°C, respectively and the enzymes were stable in the pH range from 6.0 to 8.0 and below 50°C. The enzyme activity was significantly inhibited by EDTA, Zn^{2+} and Hg^{2+}. Inquiry into the characteristics of keratinase production from these bacteria may yield useful agricultural feed processing applications.

Keywords: production, characterization, keratinase, *Paracoccus* sp. WJ-98

INTRODUCTION

Keratin is found in hair, wool, hooves, claws, beaks and feather waste, and is generated in large quantities as a byproduct of commercial poultry processing [1]. Because of a high degree of cross-linking by cystein disulfide bonds, hydrogen bonding, and hydrophobic interactions, keratin is insoluble and not degradable by proteolytic enzymes such as trypsin, pepsin, and papain [1-4]. Biodegradation of keratin is very important to improve the nutritional value of feather waste and to prevent environmental contamination.

Keratinase (EC 3.4.24.10) is a biodegradable enzyme of keratin, and also includes keratinase I (extracellular), and keratinase II and III (cell bound) [5]. In practical applications, keratinase is a useful enzyme for promoting the hydrolysis of feather keratin and improving the digestibility of feather meal, as well as having applications in waste treatment, and detergents and pharmaceuticals [1,6-10].

Microbial keratinase has been found in microorganisms such as *Aspergillus* sp., *Streptomyces* sp., *Trichophyton mentagrophytes*, *Bacillus* sp. and *Candida* sp. [1,6-10]. Lin *et al.* [8] reported that *Bacillus lichemiformis* PWD-1 grew on feathers and used them as the primary organic substrate for supplying carbon, sulfur, and energy. Biodegradation of feathers by this bacterium represents a method to improve the utilization of feathers as, for example, a feed protein. Furthermore, they also reported that the purified keratinase was monomeric and had a molecular mass of 33 kDa, and that its optimum pH and the pI were determined to be 7.5 and 7.25, respectively. Recently, Nam *et al.* [9] and Kim *et al.* [10] also reported on the isolation, and identification of keratinase-producing aerobic bacterium, and anaerobic, thermotolerant bacterium, by following the production conditions for keratinase. However, very little study has been reported on the production and characteristics of keratinase which can be used commercially in the feed industry.

This study describes the isolation of a keratinase-producing bacterium and the optimal conditions for keratinase production. Furthermore, the partial purification and characterization of the enzyme were also investigated to obtain basal data for practical application of the keratinase into feed or environmental industry.

MATERIALS AND METHODS

Materials

All chemicals used were of analytical grade. Glucose,

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DEAE-cellulase, collagen, gelatin and elastin were purchased from Sigma Chemicals Co. (St., Louis, MO., USA). Sephadex G-75 was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Keratin was purchased from Tokyo Kasei (Japan) and Bacto-tryptone, Bacto-peotone, beef extract, yeast extract, tryptone and Bacto-agar were purchased from Difco Lab. (Detroit, Michigan. USA).

Media and Culture Conditions

The basal medium used for the isolation of the keratinase-producing bacterium contained 0.5% keratin, 0.05% NH_4Cl, 0.05% KCl, 0.03% K_2HPO_4, 0.01% $MgCl_2 \cdot 6H_2O$ and 0.01% yeast extract (pH 7.5). Keratin-urea broth (1.0% keratin, 0.05% urea, 0.05% NaCl, 0.03% K_2HPO_4, 0.04% KH_2PO_4, 0.01% $MgCl_2 \cdot 6H_2O$, pH 7.5) was used in production and purification studies of the enzyme, and was cultivated in a shaking incubator or fermentor at 37°C.

Assay of Keratinase Activity

Skim-milk Plate Assay

The skim-milk plate assay was used as an alternative to the azokeratin hydrolysis assay, to screen larger numbers of the proteolytic strains [8]. One gram of skim-milk was dissolved in 98 mL of basal medium (pH 7.5), then the skim-milk plate was poured rapidly onto a clean glass plate and spread evenly. A suspension of poultry soil was inoculated onto the plate and incubated for 3 days. Clear zones around the wells were selected as strains with proteolytic activity. Keratinase activity was further identified by azokeratin hydrolysis as described below.

Assay of Keratinase Activity

Azokeratin was prepared from ball-milled feather powder [7] with sulfanilic acid and $NaNO_2$ using a method similar to that described by Tomarelli *et al.* for azoalbumin [11]. For a standard assay, 5 mg of azokeratin was added to a 1.5 mL centrifuge tube, along with 0.8 mL of 50 mM potassium phosphate buffer (pH 7.5). This mixture was agitated until the azokeratin was completely suspended. A 0.2 mL aliquot of an appropriately diluted enzyme solution was mixed with the azokeratin, and the mixture was incubated for 15 min in a 50°C water bath. The reaction was terminated by the addition of 0.2 mL of 10% trichloroacetic acid, and the mixture was filtered. The A_{450} of the filtrate was measured with a UV-2401 PC spectrophotometer (Shimadzu, Columbia, MD, USA). A control was prepared by adding trichloroacetic acid to the reaction mixture before adding the enzyme solution. Without knowing the molar extinction coefficient for azopeptides, we defined 1 U of keratinase activity as an 0.01 increase in the A_{450} after 15 min in the test reaction, when compared with the control reaction.

Partial Purification of the Keratinase

The culture broth was centrifuged (5000 × g, 10 min) to remove residual undegraded keratin, bacterial cells and other particles. The filtrate was adjusted to 30~70% precipitation with saturated ammonium sulfate. The pellet was resuspended and dialyzed overnight against a potassium phosphate buffer (pH 7.5), and then concentrated. The crude concentrated keratinase solution was applied to a column of DEAE-cellulose (2.5 by 60 cm) at 4°C. Approximately 12.3 mg of protein was loaded on the column. Fractions were assayed for keratinase activity using the azokeratin hydrolysis test. Active fractions were pooled and concentrated using PEG at 4°C. The purification of keratinase continued using a Sephadex G-75 column (1.5 by 90cm) at 4°C, and equilibration and elution were carried out at 4°C with a 50 mM potassium phosphate buffer (pH 7.0) at a flow rate of 0.3 mL/min. Fractioned volumes of 3 mL were collected. Following protein elution, the A_{280} of each fraction was monitored. Protein fractions were assayed for keratinase activity with azokeratin, and then the keratinase activity fractions were pooled and concentrated [8].

RESULTS AND DISCUSSION

Isolation and Identification of Keratinase-producing Bacterium

Nine hundred eighty different strains of bacteria were isolated from 40 poultry soil samples from the Daejeon and Chungnam areas. Since the WJ-98 strain exhibited the highest keratinase activity, it was selected for examination as a new microorganism that produces keratinase.

Morphological, biochemical and cultural characteristics of the WJ-98 strain are summarized in Table 1, and Fig. 1. WJ-98 is a short, rod shaped bacterium of 25 × 1.0 μm that is gram negative, and non-motile. The Voges-Proskauer test was negative, and the oxidase, catalase and indole tests were all positive. GC content was 65 mol%.

A phylogenetic analysis based on 16S rRNA sequence was finally used to identify the strain WJ-98. A 16S rRNA was amplified by the colony PCR using two universal primers (9F, 5'-AGA GTT TGA TCC TGG CTC AG; 926R, 5'-CCG TCA ATT CCT TTR AGR TT) described by Yim *et al.* [12]. The PCR product was purified using a QIAquick PCR purification kit (Qiagen) and them determined its nucleotide sequences. Its homology was investigated by using BLAST program of NCBI. Its 16S rRNA sequences revealed that it was close in proximity to *Paracoccus* sp. (similarity to *Paracoccus* sp.; 99.9%) (Fig. 2). Based on its microbial characteristics and 16S rRNA sequences, WJ-98 was identified using Bergey's manual to be *Paracoccus* sp. WJ-98. *Bacillus* sp., *Candida* sp., *Streptomyces* sp. and *Aspergillus* sp. are known to be producers of microbial keratinase and to act as feather digestion agents [1,6-10]. However, the keratinase-producing characteristics of *Paracoccus* sp. WJ-98 are reported for the first time in this paper.

Paracoccus sp. WJ-98 was also found to be a thermophilic bacteria which could grow up to 50°C and showed no resistance to several antibiotics, such as penicillin and chloramphenicol, tetracycline and streptomycin except ampicillin at 100 μg/mL.

Table 1. Morphological, biochemical and cultural characteristics of the selected strain, WJ-98

Test	Results
Morphological characteristics	
Shape and size	Rod, straight, 2.5 × 1.0 μm
Gram staining	Negative
Mobility	Non-motile
Spore	–
Flagella	–
Biochemical and cultural characteristics	
Methyl red test	+
Voges-Proskauer test	–
Catalase test	+
Oxidase test	+
Indole test	+
Ammonia test	–
H_2S test	–
Assimilation of citrate, glucose	+
fructose, sucrose	+
acetate, fumarate	+
rhamnose, mannitol	+
ethanol	+
Hydrolysis of gelatin	+
Reduction of nitrate	–
Temperature for growth	50°C (optimum temp. 37°C)
pH for growth	pH 5.0 ~ 9.0 (optimum pH 7.5)
Oxygen requirement	Aerobic
Antibiotic resistance	
Ampicillin	+
Penicillin	–
Chloramphenicol	–
Steptomycin	–
Kanamycin	–
Tetracycline	–
G+C content	65 mol%

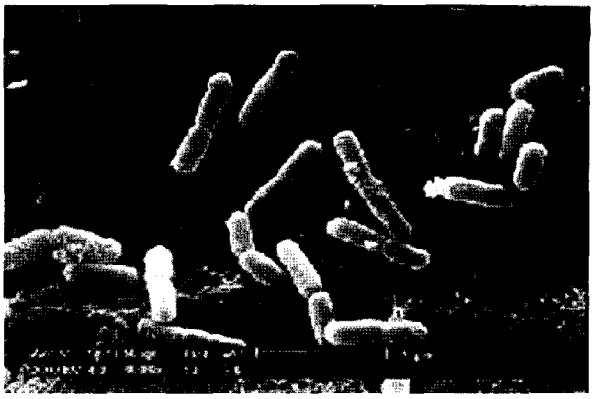

Fig. 1. Scanning electron micrograph of the selected strain, WJ-98.

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31 catgcaagtcgagcgacaccttcgggtctagcggcggacgggtgagtaacgcgtgggaac 90
91 gtgcccttctctacggaatagccccgggaaactgggagtaataccgtatacgccctttgg 150
151 gggaaagatttatcggagaaggatcggcccgccgttggattaggtagttggtggggtaat 210
211 ggcccaccaagccgacgatccatagctggtttgagaggatgatcacccacactgggactg 270
271 agacacggcccacactcctacgggaggcagcagtggggaatcttagacaatgggggcaac 330
331 cctgatctagccaatgccgcgtgagtgatgaaggccttagggttgtaaagctctttcagc 390
391 tgggaagataatgacggtaccagcagaagaagcgcccggctaactccgtgccagcagccg 450
451 cggtaatacggagggggctagcgttgttcggaattactgggcgtaaagcgcacgttaggc 510
511 ggactggaaagtcagaggtgaaatcccagggctcaaccttggaactgcctttgaaactat 570
571 cagtctggagttcgaagagaggtgagtggaattccgagtgtagaggtgaaattcgtagat 630
631 attcggaggaacaccagtggcgaaggcggctcactgcgctcgatactgacgctgaggtgc 690
691 gaaagcgtggggagcaaacaggattagataccctggtagtccacgccgtaaacgatgaat 750
751 gccagacgtcggcaagcatgcttgtcggtgtcacacctaacggattaagcataccgc 807
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Fig. 2. Nucleotide sequence of the partially amplified 16S rRNA gene from *Paracoccus* sp. WJ-98 by PCR.

Production Conditions of the Keratinase

The effects of nitrogen sources on enzyme production are shown in Table 2. About 61 U/mL^{-1} of keratinase was produced by the addition of urea into a basal medium containing 0.5% keratin, 0.03% K_2HPO_4, 0.04% KH_2PO_4 and 0.01% $MgCl_2 \cdot 6H_2O$. However, the addition of other nitrogen sources such as yeast extract, peptone and tryptone were not effective in producing keratinase. These results contrasted with those reported for *Pseudomonas* sp. KP-364 [13] and alkalophilic *Bacillus* sp. No. AH 101 [14] in which soybean meal, malt extract and yeast extracts encouraged good growth and keratinase production; and in *B. licheniformis* PWO-1 [1], which also responded well to the addition of NH_4Cl.

The effects of concentrations of urea and keratin on keratinase production were also investigated. As shown in Table 2, the enzyme showed a maximum production of 91 U/mL at 1.0% keratin supplement with 0.05% urea.

Meanwhile, the effects of carbon sources on the enzyme

Table 2. Effects of nitrogen sources on the production of keratinase from *Paracoccus* sp. WJ-98

Nitrogen sources[1)]	Cell growth (A_{660})[2)]	Keratinase activity (U/mL)
Yeast extract	n.d[3)]	n.d
Peptone	0.87	n.d
Tryptone	0.85	3
Beef extract	0.56	11
Corn steep liquor	0.95	11
Casamino acid	0.52	14
Urea	0.99	61
NH_4Cl	0.82	8
NH_4Cl_3	0.83	10
$(NH_4)_2SO_4$	0.98	16
KNO_3	0.89	12
$NaNO_3$	0.98	8
Ammonium citrate	1.21	8
Ammonium oxalate	1.07	13
Keratin 0.1% + urea 0.005%	0.50	19
0.01%	0.57	37
0.05%	0.50	31
0.5% + urea 0.005%	0.46	12
0.01%	0.97	64
0.05%	0.87	62
1.0% + urea 0.005%	0.30	16
0.01%	0.73	49
0.05%	0.53	91
1.0%	0.21	25
Control	0.79	47

[1)] All nitrogen sources were added to 0.05%
[2)] Cultivation was carried out for 84 h at 37°C in the basal medium containing 0.5% keratin, 0.03% K_2HPO_4, 0.04% KH_2PO_4, 0.01% $MgCl_2 \cdot 6H_2O$
[3)] n.d; not determined

production were examined using the keratin-urea medium. No sugars were effective in keratinase production (data not shown). However, Chon and Kwon. [13], Yu *et al.* [15] and Page and Stock. [16] reported that the addition of glucose, raffinose and galactose increased its growth and keratinase production. Considering the optimal medium composition for keratinase production, we concluded that keratinase production by *Paracoccus* sp. WJ-98 was very economical because expensive organic nitrogen sources as yeast extracts and peptone were not used in keratinase production and recovery is also very easy due to extracellular keratinase.

The effects of the medium's initial pH on keratinase production were examined in media of various pH. As shown in Fig. 3, the highest activity rate was observed in cultures grown on a medium with an initial pH of 7.5, and significant enzyme was also produced at pH 6.0 and 9.0. This result was similar to that of *Bacillus licheniformis* (pH 7.5) [1]; however it was different from that of *Pseudomonas* sp. KP-364 (pH 6.5) [13]. The optimal temperature for enzyme production was 37°C, which is comparable to that of *Streptomyces pactum* DSH 40530 (30°C), *Pseudomonas* sp. KP-364 (30-40°C) [13] and *Dermatophilus congolensis* (37°C) [17] (data not shown).

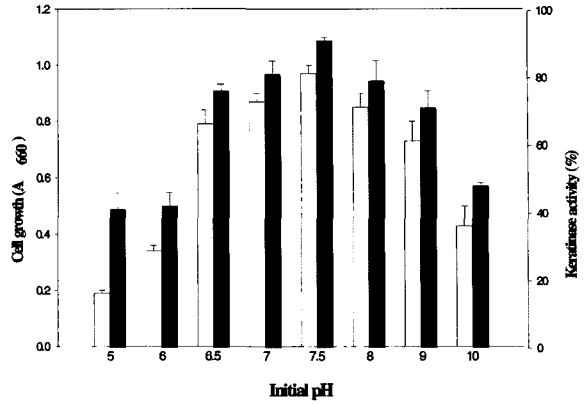

Fig. 3. Effects of the medium's initial pH on keratinase production from *Paracoccus* sp. WJ-98. Keratinase activity and cell growth were measured after the cultivation in keratin-urea medium containing 1.0% keratin, 0.05% urea, 0.03% K_2HPO_4, 0.04% KH_2PO_4 and 0.01% $MgCl_2 \cdot 6H_2O$. Symbol; □: growth ■: keratinase activity.

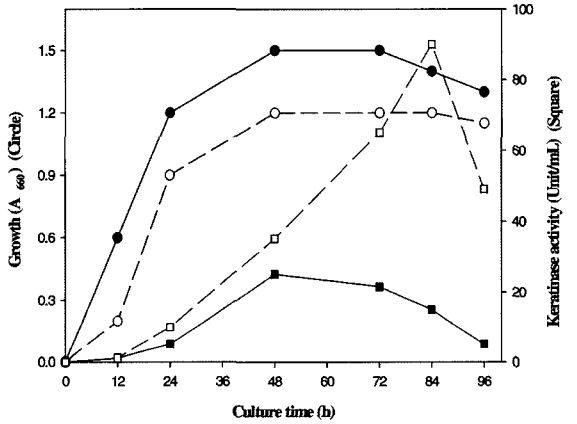

Fig. 4. Effects of flask culture (--) and batch culture by fermentor (—) on keratinase production from *Paracoccus* sp. WJ-98.

The effects of cultivation modes on keratinase production were investigated by flask-shaking culture and batch culture using a jar fermentor (7 L) under the optimum conditions described above. Enzyme production in the flask culture (stationary phase) increased to maximum levels of cell growth and maximum productivity after 84 h and its keratinase activity was measured at 90 U/mL (Fig. 4). However, the maximum cell growth and keratinase

Table 3. Effects of metal ions and chemical compounds on the activity of keratinase from *Paracoccus* sp. WJ-98

Reagents[1)]	Relative activity (%)
K^+	96.1
Na^+	114.5
Li^+	106.6
Cu^{2+}	88.2
Ca^{2+}	92.1
Fe^{2+}	69.7
Mg^{2+}	84.2
$Mn2^+$	57.9
Zn^{2+}	39.5
Hg^{2+}	19.7
$Al2^+$	59.2
EDTA	49
EDTA-Na^{2+}	15
EDTA-Na^+	19
EDTA-Li^+	18
EDTA-K^+	51
EDTA-Ca^+	54
Sodium thiosulfate	56.6
PMSF	103
L-cystein	69
1,10-phenanthronin	49
potassium cyanide	82
control	100

[1)] All of the cations were added as chlorides except that Mn^{2+}, Cu^{2+}, Zn^{2+}, Al^{2+} and Fe^{2+} were added as sulfates. The enzyme solutions containing 1 mM (all reagents except K^+ and Na^+) to 25 mM (K^+, Na^+) of cations were preincubated at 50°C, 15 min and then the residual enzyme activity was measured.

production in the batch culture was reached after 24 h and 48 h (25 U/mL), respectively. Even though growth and keratinase production were faster in the batch culture with the fermentor than those of the flask culture, keratinase production of the batch culture was about 4 folds lower than that of the flask culture. From the results of protein analysis of fermentation broth after fermentation, it was probably due to more faster exhaustion of keratin in the fermentor culture than in the flask culture. Therefore, the flask culture was considered to be good for mass production of keratinase from *Paracoccus* sp. WJ-98. However, its keratinase productivity of 360 U mL^{-1} h^{-1} was about 3 folds lower than that of *Pseudomonas* sp. KP-364 (1,270 U mL^{-1} h^{-1}) [13].

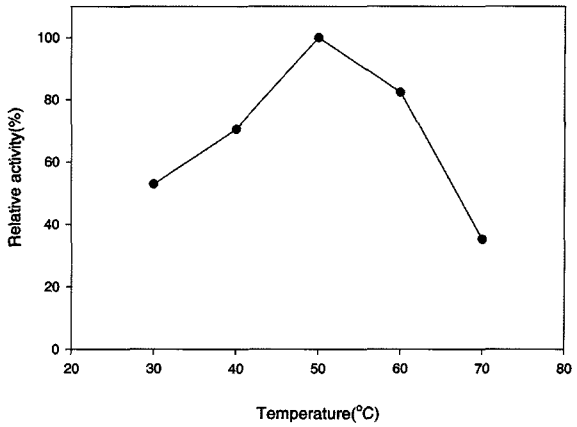

Fig. 5. Optimal reaction temperature of keratinase from *Paracoccus* sp. WJ-98.

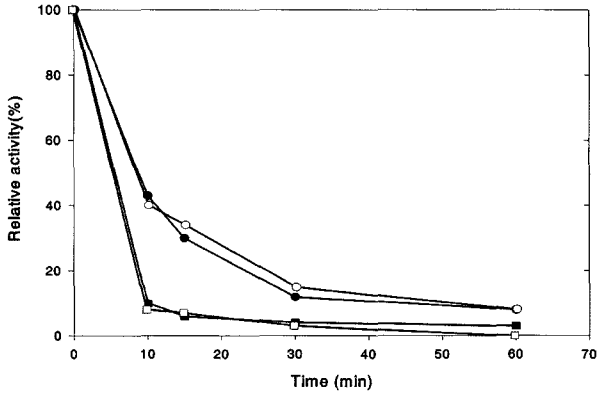

Fig. 6. Thermal stability of keratinase from *Paracoccus* sp. WJ-98. ● : 50°C, ○ : 60°C, ■ : 70°C, □ : 80°C

Characteristics of the Keratinase

Keratinase from *Paracoccus* sp. WJ-98 was partially purified by ammonium sulfate precipitation (30%~70% saturation), followed by ion exchange chromatography on DEAE-cellulose, and then gel filtration chromatography on Sephadex G-75.

The optimum temperature for the enzyme reaction was found to be 50°C, and about 40% of its activity was retained at 60°C during 20 min of incubation (Figs. 5,6). These results were similar to those of the keratinase from *Bacillus licheniformis* [8].

The effects of pH on the enzyme activity and pH stability were examined in a range of pH 5.0~9.0 (Fig. 7). The optimum pH was shown to be 6.5, which was more acidic than that of *Bacillus licheniformis* (pH 7.5) [8]. Furthermore, keratinase was also stable in the range of pH 6.0~8.0.

Na^+, K^+ and Li^{2+} showed no inhibitory effects on enzyme activity at 25 mM or 1.0 mM final concentration. However, Hg^{2+}, Zn^{2+}, EDTA and 1,10-phenanthronin strongly inhibited the enzyme activity (Table 3). The mixture of EDTA (1 mM) and Na^+ (25 mM), Li^{2+} (1 mM)

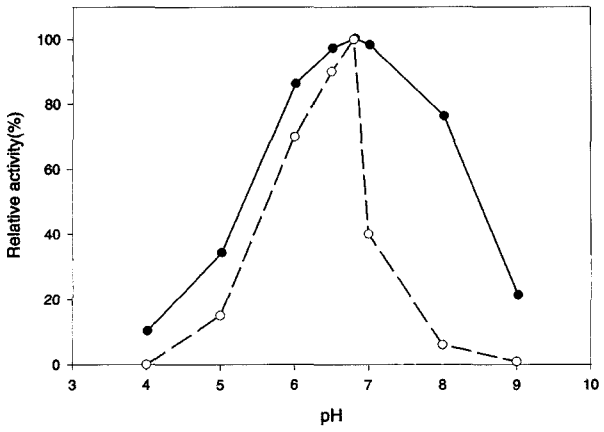

Fig. 7. Optimal pH (--) and pH stability (—) of keratinase from *Paracoccus* sp. WJ-98. The reaction mixture containing 0.8 ml of 0.6% keratin in 50 mM of various buffer (pH 4.0 - 9.0) and 0.2 mL of the enzyme was incubated for 15 min at 50°C (optimal pH). The enzyme (0.1 mL) was mixed with 0.1 mL of 50 mM of various buffer (pH 4.0~10.0), preincubated for 1 hr. The relative activity of the treated enzymes was assayed under the standard assay condition (pH stability). Citrate buffer: pH 4.0~6.0, phosphate buffer: pH 6.0~8.0, Tris buffer: pH 8.0~9.0

also significantly inhibited the enzyme activity. Meanwhile, PMSF and potassium cyanide did not affect the enzyme activity.

Extensive efforts were made to adapt crude keratinase from *Paracoccus* sp. WJ-98 to digest native feather keratin. The digestivity of feather keratin by the enzyme increased slightly, but the degradation of non-steam-treated feathers was unsuccessful. Therefore, we inferred that keratinase from *Paracoccus* sp. WJ-98 may be more applicable in the preparation of feed additives to improve the digestibility of feather meal in the poultry farm, than in the treatment of feathers as environmental pollutants. Further investigation on pretreatment conditions which enable non-steam-treated feathers to be degraded effectively by this enzyme are underway.

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