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FEATHER MEAL AND RICE HUSK ENHANCED KERATINASES PRODUCTION BY *BACILLUS LICHENIFORMIS* YJ4 AND CHARACTERS OF PRODUCED KERATINASES

Hsin-Hung Lin* and Li-Jung Yin**

Key words: *Bacillus licheniformis*, feather, rice husk, keratinase, character.

ABSTRACT

A feather-degrading bacterium with high keratinase activity was isolated and identified as *Bacillus licheniformis* YJ4. After 72 h incubation in a medium (0.5% feather meal, 0.05% NH_4Cl and NaCl, 0.04% K_2HPO_4, 0.03% KH_2PO_4, 0.01% $MgCl_2$ and yeast extract, 0.1% rice husk) at 37°C, 2 keratinases (keratinase I and II) were purified to electrophoretical homogeneity by CM sepharose and Sephadex G-75 chromatographs. They were with molecular masses (*M*) of 35.5 and 32.8 kDa, isoelectric point (p*I*) of 6.63 and 6.50, respectively, and stable at pH 6.0-10.0 and 10-50°C. The optimal pH and temperature were similar, at 9.0 and 60°C, respectively. According to the effect of metal, inhibitor and reducing agent, and previous studies, the purified keratinases I and II were considered to be cysteine and serine proteases, respectively.

I. INTRODUCTION

Keratin, major component of feathers, is rich in disulfide bonds and also very stable and highly resistant to proteolysis by pepsin, trypsin, papain, etc. [10]. Currently, most of feathers from poultry industry are processed into feather meals by using high pressure and temperature. These dramatic conditions are time-consuming and high cost, and usually lower the nutritional value [26]. Because of being able to hydrolyze insoluble keratin substrate, the bacterial proteases has attracted many scientists and been well studied on the hydrolysis of hair and feather keratins [27].

Williams *et al*. [28] isolated a feather-degrading bacterium, *Bacillus licheniformis* PWD-1. According to their experiments on chicken, those fed by biodegraded feathers had better growth than those fed by untreated one or commercial feather diet [27, 28]. According to Williams *et al*. [27], the hydrolyzed feathers are still used as part of nitrogen source for animal feed, even though their high cost in the production of keratinase. Some other studies further indicated that keratin hydrolysates were also good organic fertilizers, materials for edible film processings and limited amino acids supplement for health foods and pet foods [10].

Poultry industry produces a large quantity of by product, feather, each year and greatly impacts our environment [27]. Economical production of keratinases and simplified feather proteolysis are, accordingly, of necessity for poultry industry [19]. Accordingly, recycling these feathers has long been concerned by many scientists and governments worldwide. Keratinases are considered to be able to proteolyze feather and can be produced by many microorganisms such as bacteria, actinomyces and fungi [10]. This study aimed to isolate the keratinase-producing bacterium, and further optimize the cultivation medium. Keratinases produced by isolated strain were also purified and characterized.

II. MATERIALS AND METHODS

1. Materials

Feather meal (100 meshes) from Arbor Acres Chicken and rice husk were pre-treated and kindly provided from Charoen Pokphand Enterprise (Indonesia) Co., LTD. All media for bacteria cultivation were from Difco Laboratories (BD Co., MD, USA). Azokeratin was prepared in our laboratory according to Riffel *et al*. [19]. All metals, inhibitors and reducing agents were the products of Sigma-Aldrich Inc. (St. Louis, MO, USA).

2. Screening of Keratinase-Producing Bacteria

The feather degrading bacterium was screened from decomposed chicken feathers from a local poultry slaughter house in southern Taiwan. Ten % of sample suspended in sterile phosphate buffered saline (PBS, 10 mM potassium

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phosphate buffer, 150 mM NaCl, pH 7.4) was inoculated and incubated in a 1.0% feather meal broth (pH 7.5) at 37°C for 7 days. To a medium (feather meal broth, FMB) containing 1.0% feather meal, 0.05% of NH_4Cl and NaCl, 0.04% K_2HPO_4, 0.03% KH_2PO_4, 0.01% of $MgCl_2$ and yeast extract (pH 7.5), 10% of the resulted broth was inoculated and incubated at 37°C for 3 days. The resulted broth was then spread on a feather meal agar plate (FMA) (FMB + 1.5% agar) and incubated at 37°C for 3 days. The colonies with clear zone (indicating those with feather hydrolyzing ability) on FMA were selected and inoculated into a fresh FMA. After 3 days incubation at 37°C, the colony with highest feather hydrolyzing ability was picked up, re-activated in TSB (Tryptic soy broth) and subjected to identification. The isolated strain was stored at -80°C in TSB with 20% (v/v) glycerol for the further study.

3. Bacterial Identification

API identification kits were used for the identification. Bacterial isolate was cultivated on nutrient agar (NA) plates at 37°C for 3 days. The colony was re-suspended in 2 mL of sterile distilled water and inoculated on 50 CHB and 20 E API strips (BioMérieux, France) according to manufacturer's instruction. After 16, 24, 40 and 48 h incubation at 37°C, the strips were removed and compared with API identification index and database.

The sequence of 16s rDNA of isolated strain was determined after genomic DNA extraction and PCR amplification as described by Thys *et al*. [25]. Comparison of the sequence with homologous strains in GenBank was performed using Basic Local Alignment Search Tool (BLAST). The phylogenetic tree was inferred from BLAST at National Center for Biotechnology Information (NCBI).

4. Production of Keratinase

To optimize medium composition for the production of keratinase, the inoculum, 1 mL of isolated strain in TSB with 20% (v/v) glycerol from -80°C was added to 10 mL of TSB and incubated at 37°C overnight (10^8 CFU/mL). One mL of the activated isolated strain was transferred to 100 mL various media (FMB with various amounts of carbon or nitrogen sources) in a 250 mL flask. The isolated strain in these media was incubated under aerobic conditions at 37°C with 150 rpm shaking. The viable cell counts (CFU/mL), keratinase activity and pH were measured during incubation. The optimal medium was employed for the production of keratinase.

5. Purification of Keratinase

The cultured broths were filtered through glass wool to remove the un-degraded feathers and then through a 0.45-μm sterilized membrane (Gelman Sciences, Ann Arbor, MI) to remove bacterial cells. After being concentrated by membrane ultrafiltration (cutoff: 5,000) with an Amicon ultrafiltration system (Amicon Div., W. R. Grace and Co., Beverly, MA, USA) and dialyzed against a buffer containing 50 mM potassium phosphate buffer (pH 6.0, buffer A) overnight, the crude keratinase was chromatographed on a CM sepharose Fast Flow (2.6 × 30 cm) which was equilibrated with buffer A. The proteins were eluted by 500 mL of buffer A with a linear gradient of 0.0 to 0.5 M NaCl at a flow rate of 0.5 mL/min. Fractions with keratinase activity were collected and subjected to Sephadex G-75 chromatography (1.6 × 70 cm). The enzymes were eluted with a buffer containing 25 mM potassium phosphate, 100 mM NaCl (pH 7.5, buffer B) at a flow rate of 0.5 mL/min. Eluted enzymes were collected at 4°C with 2.0 mL/tube. Fractions with keratinase activity were pooled and concentrated using an Amicon ultra-filtration system with Diaflo ultrafilters (cutoff: 10,000, Amicon Div., W. R. Grace and Co., Beverly, MA, USA) at 4°C.

6. Assay of Keratinase Activity

Keratinase activity was determined by measuring the hydrolysis ability on azokeratin according to Sangali and Brandelli [22]. Azokeratin was prepared as described by Riffel *et al*. [19]. Briefly, 200 μL of enzyme extract was added to 800 μL of azokeratin (5 mg/mL) in 50 mM phosphate buffer (pH 7.5). After 60 min reaction at 50°C, equal volume of 15% trichloroacetic acid (TCA) solution was added to stop the reaction. Absorbance at 450 nm was then measured after 5 min centrifugation at 10,000 × g. One unit of activity was defined as the amount of keratinase that caused an increase in absorbance of 0.01 at 450 nm within 60 min reaction at 50°C.

7. Determination of Protein Concentration

Protein concentrations were determined by dye binding method [4] using bovine serum albumin as standard.

8. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The SDS-PAGE analysis was carried out in a 12.5% polyacrylamide gel according to Laemmli [12]. Ten μg of enzyme samples was applied on each cell of gel. After electrophoretic running, gels were stained with Coomassie blue R-250 [17]. Low molecular weight calibration kit (GE Healthcare Bio-Sciences Corp., MA, USA) was used as markers [Phosphorylase b subunit (97 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean inhibitor (20.1 kDa), and α-lactalbumin (14 kDa)].

9. Isoelectric Point (p*I*)

The p*I* of purified enzymes were measured using isoelectric focusing (IEF) on a Pharmacia Ampholine PAG plate (Multiphor II electrophoresis unit, GE Healthcare BioSciences Corp., MA, USA) (pH 3.5-9.5) and compared with standards from an IEF calibration kit according to the manufacturer's instruction. Trypsinogen (9.30), lentil lectin basic subunit (8.65), lentil lectin middle subunit (8.45), lentil lectin acidic subunit (8.15), myoglobin basic subunit (7.35), myoglobin acidic subunit (6.85), human carbonic anhydrase B (6.55), bovine carbonic anhydrase B (5.85), β-lactoglobulin A (5.20), soybean trypsin inhibitor (4.55) and amyloglucosidase (3.50)

were used as markers (GE Healthcare BioSciences Corp., MA, USA).

10. N-terminal Amino Acid Sequence Analysis

Purified keratinases were subjected to SDS-PAGE analysis and then electro-transferred onto polyvinylidene difluoride membrane (PVDF). Proteins were stained with Coomassie blue R-250 after electrophoresis. N-terminal amino acid sequences were analyzed by Edman degradation [7] after being electro-transferred onto PVDF.

11. Effects of pH and Temperature

The effects of pH and temperature on keratinase activity were determined using azokeratin as substrate. The optimal pH was determined by measuring the activity of purified keratinases at pH 3.0-11.0 (pH 3.0-7.0 using 20 mM citrate buffer, pH 6.0-9.0 using 20 mM sodium phosphate buffer, pH 8.0-11.0 using 20 mM sodium carbonate buffer), while the optimal temperature of purified keratinases in 20 mM sodium phosphate buffer (pH 7.5) was measured at temperatures from 10 to 90°C according to Sangali and Brandelli [22].

The pH stability was determined by incubating keratinases in various buffers with pH 3.0-11.0 (pH 3.0-7.0 using 20 mM citrate buffer, pH 6.0-9.0 using 20 mM sodium phosphate buffer, pH 8.0-11.0 using 20 mM sodium carbonate buffer) at 25°C for 60 min. The residual activity was determined according to Sangali and Brandelli [22].

The thermal stability was determined by incubating keratinases in 20 mM sodium phosphate buffer (pH 7.5) at temperatures from 10° to 90°C for 60 min. The residual activity was then determined according to Sangali and Brandelli [22].

12. Effects of Metal Ion, Reducing Agent or Inhibitor

Purified keratinases in 20 mM Tris-HCl buffer (pH 7.5) with metal ions (Li^{+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Ni^{2+}, Zn^{2+}, Fe^{3+}), reducing (thiol) agents such as dithiothreitol (DTT), glutathione (GSH), β-mercaptoethanol (β-Me) and other reagents or inhibitors such as ethylene diamine tetraacetic acid (EDTA), N-ethylmaleimide (NEM), *p*-chloromercuribenzoate (*p*CMB), phenylmethyl sulfonyl fluoride (PMSF) and N-toyl-L-lysine chloromethyl ketone (TLCK), etc. were incubated at 25°C for 30 min. The residual activity was measured according to Sangali and Brandelli [22].

13. Statistical Analysis

Duncan's multiple range tests was employed to determine the significance of difference among treatments. For each treatment, 3 measurements were used for calculation. Values were considered to be significantly different when $p < 0.05$.

III. RESULTS AND DISCUSSION

1. Screening and Identification of Keratinase-Producing Bacteria

The keratinase-producing bacteria, survived in 1.0% of feather meal broth after 7 days incubation at 37°C, were isolated from feather dunghill. After the strains were transferred to FMA, only 6 colonies with feather degrading abilities were observed from the total 50 colonies (data not shown). Strain with the highest hydrolytic activity was subjected to identification.

Table 1. Effects of extra nitrogen and carbon sources on the production of keratinase from *Bacillus licheniformis* YJ4.

Media	Keratinase (units/mL)
Control[#]	80 ± 4
Casein*	50 ± 2
Glucose*	27 ± 3
Rice husk*	124 ± 2
Peptone*	33 ± 2
Soytone*	30 ± 4
Soya meal*	55 ± 3
Starch*	3 ± 1
Surcose*	30 ± 2
Tryptone*	20 ± 4

[#]Control: cultivated in FMB (1.0% feather meal, 0.05% of NH_4Cl and NaCl, 0.04% K_2HPO_4, 0.03% KH_2PO_4, 0.01% of $MgCl_2$ and yeast extract, pH 7.5).

*Cultivated in FMB with 0.1% of extra ingredient after 72 h incubation at 37°C.

According to the bacterial identification kit (ABI 50 CHB and ABI 20E) and 16s rDNA sequencing (data not shown), this strain was identified as *Bacillus licheniformis* YJ4, being a catalase-positive, oxidase-negative, endospore-forming, and Gram-positive bacterium. The alignment of 16s rDNA by BLAST from GenBank indicated almost 99.6% sequence similarity with that from *Bacillus licheniformis* N8 (DQ350834) and 99.0% similarity with that from *Bacillus* sp. DCA-X (DQ305286). Keratinases from *Bacillus* species have long been studied and found that they could hydrolyze feathers [13, 15].

2. Conditions for the Growth of Isolated *Bacillus licheniformis* YJ4

Among the extra nitrogen or carbon sources used, high level of keratinase was obtained when it was grown in FMB containing 0.1% rice husk after 72 h incubation at 37°C. As shown in Table 1, the other extra ingredients actually decreased keratinase production by *Bacillus licheniformis*. In order to optimize the concentration of rice husk on the production of keratinase, 0.05 to 1.0% (w/v) of rice husk was added to FMB. Among various rice husk contents in FMB broth, keratinase increased significantly on medium with 0.1% (w/v) rich husk after 72 h incubation at 37°C (124 units/mL, $p < 0.05$) (Fig. 1(a)). Significant improvement in yield of keratinase was observed on the medium with extra 0.5% (w/v)

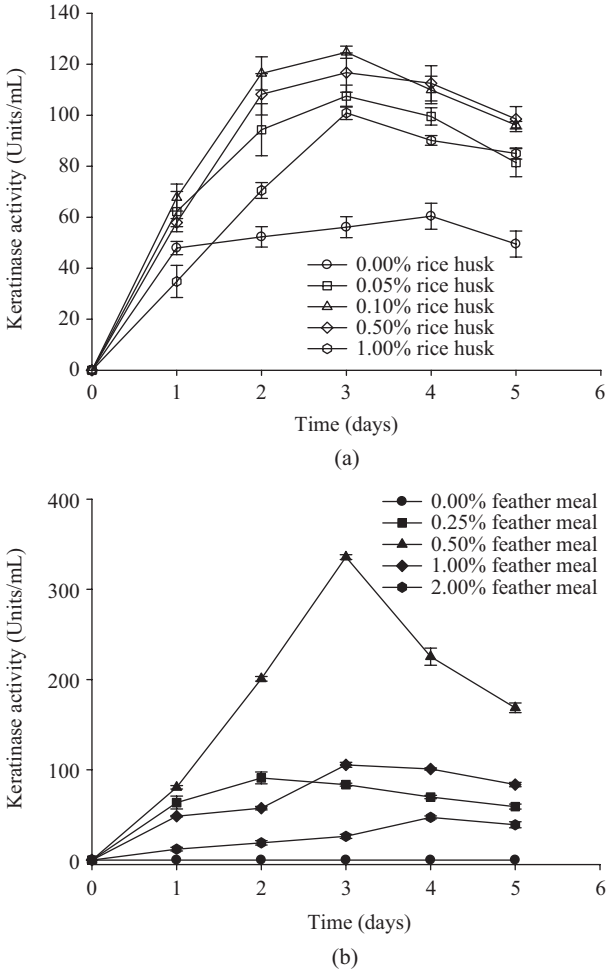

Fig. 1. Effect of rice husk (a) and feather meal content (b) on keratinase production from *Bacillus licheniformis* YJ4 during 5 days incubation at 37°C (A: Cultivated in FMB broth (1.0% feather meal, 0.05% of NH_4Cl and NaCl, 0.04% K_2HPO_4, 0.03% KH_2PO_4, 0.01% of $MgCl_2$ and yeast extract; pH 7.5) with various rice husk contents; B: Cultivated in various feather meal contents of FMB broth with 0.1% rice husk).

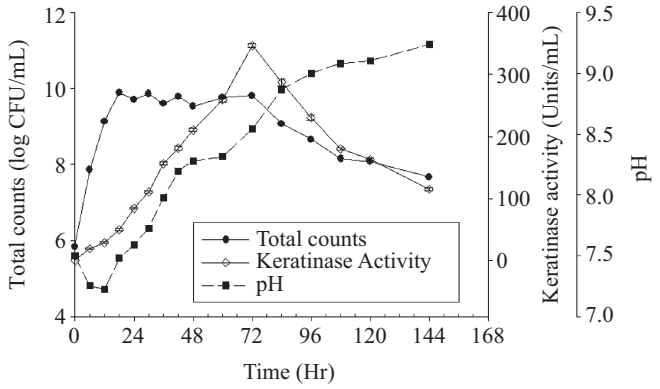

Fig. 2. Changes in total count, keratinase activity and pH of *Bacillus licheniformis* YJ4 during 6 days incubation at 37°C.

feather meal (348 units/mL) after 72 h incubation at 37°C (Fig. 1(b)). Medium containing 0.5% feather meal, 0.1% rice husk, 0.05% of NH_4Cl and NaCl, 0.04% K_2HPO_4, 0.03% KH_2PO_4, and 0.01% of $MgCl_2$ and yeast extract (pH 7.5) was, therefore, used for further studies.

The yield of keratinase from this strain was influenced by physiological and natural substrate, *i.e.* rice husk. It was also related to the growth of the microbial strain. From the data obtained in this study, the soytone, tryptone or peptone nutrients could be replaced by rice husk and feather, which are by-products from crop and poultry industries, and could also obtain maximum keratinase production. The use of these alternative low cost nutrients will substantially reduce the production cost for this protease and further benefit the feather industry.

As shown in Fig. 2, after 12 h incubation, the culture entered stationary phase and keratinase production was greatly increased, with maximum of 350 U/mL being obtained at 72 h. After 72 h, both bacterial count and keratinase activity were declined, while the broth of pH continuously increased up to 9.24. This phenomenon suggested that the maximum proteases production was at late stationary phase, which was similar to that obtained by Altalo and Gashe [1]. These results also corresponded with previous study indicating that neutral or alkaline cultivation were suitable for keratinase production [23]. Therefore, broth of *Bacillus licheniformis* YJ4 after 72 h incubation at 37°C was collected for further purification and characterization of keratinases.

3. Purification of Keratinases

Keratinases were purified by ultrafiltration, CM sepharose Fast Flow ion-exchange and Sephadex G-75 chromatographs (Table 2). A specific activity of 800 units/mg was obtained after passing through a 0.45-μm membrane. Two peaks with keratinase activity on CM Sepharose Fast Flow chromatography were observed and designated as keratinase I and II, respectively (Fig. 3). Since some very minor contamination was still observed at this stage, the resulted samples were, therefore, further passed through Sephadex G-75 chromatography (Fig. 4). Both keratinases were purified to electrophoretical homogeneity with specific activity of 876 and 1,060 units/mg and purification fold of 1.96 and 2.37, respectively (Table 2). Although some studies indicated that using affinity chromatography or hydrophobic interaction chromatography could increase the yield and recovery of keratinase [3, 9], they did not affect the yield in this study (data not shown).

4. Molecular Mass (*M*), Isoelectric Point (*pI*) and N-terminal Amino Acid Sequence of Keratinases

According to Sephadex G-75 chromatography and SDS-PAGE (Fig. 5), both purified keratinase I and II, with molecular masses (*M*) of 35.5 and 32.8 kDa, and Isoelectric point (p*I*) of 6.63 and 6.50 (data not shown), respectively, were monomer. The *M* of both keratinases were similar to those

Table 2. Summary of the purification of keratinase from *Bacillus licheniformis* YJ4.

Procedures	Total Protein (mg)	Total Activity (unit)	Specific Activity (unit/mg)	Recovery (%)	Purification (fold)
Crude enzyme	186	83,162	447	100.00	1.00
Ultra-filtration	129	79,800	619	95.96	1.38
CM Sepharose					
Keratinase I	3.03	2,595	856	3.12	1.92
Keratinase II	9.7	9,840	1,014	11.83	2.27
Sephadex G-75					
Keratinase I	2.78	2,436	876	2.93	1.96
Keratinase II	9.05	9,592	1,060	11.53	2.37

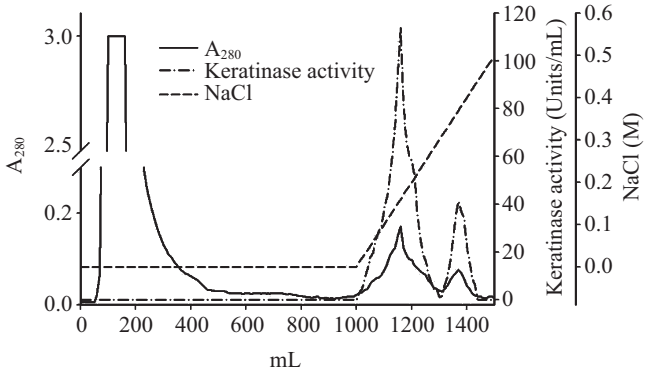

Fig. 3. Chromatogram of keratinases on CM Sepharose Fast Flow (The crude enzyme solution was concentrated with 50 mM potassium phosphate buffer, pH 6.0 (buffer A) using an Amicon ultrafiltration. The resulted sample was applied onto CM Sepharose Fast Flow which was previously equilibrated with buffer A. After being washed with buffer A, the column was then eluted using a linear gradient of 0 to 0.5 M NaCl in buffer A. Fractions of 5 mL were collected at a flow rate of 0.5 mL/min. ——,A A_{280}; – – –, NaCl; – • – , keratinase activity).

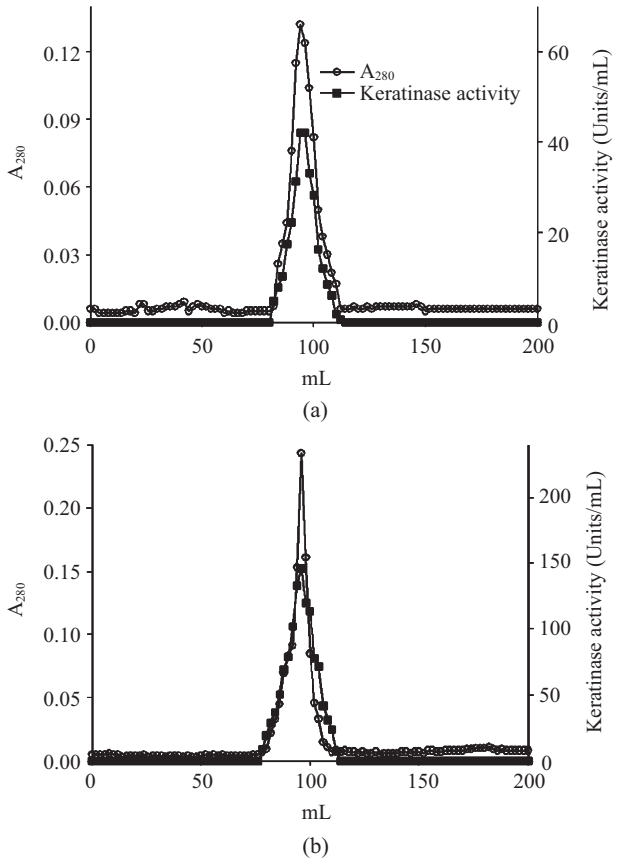

Fig. 4. Elution profile of keratinase I (a) and II (b) on a Sephadex G-75 chromatography. Elution was performed using buffer B at a flow rate of 0.5 mL/min. (——) A_{280}; (– • –) keratinase activity.

from *Bacillus licheniformis* PWD-1, *Strepomyces pactum* DSM 40530, and *Doratomyces microsporus* (30-35 kDa) [3, 9, 15].

The N-terminal amino acid sequence of keratinase II was DINGGGAT, which was completely the same as that of subtilisins. Accordingly, it was considered to be the subtilisin family of serine protease [1]. On the contrary, the N-terminal amino acid sequence of keratinase I was AQTVPYGI, which was completely different from those of other *Bacillus* strains in database of NCBI.

5. Effects of pH and Temperature

Both keratinases had similar optimal pH and temperature at 9.0 and 60°C, respectively, and were stable at pH 6.0~10.0 and 10~50°C (Figs. 6 and 7). Keratinases from bacteria, actinomycetes and fungi had optimal pH from neutral to alkaline [2, 8, 11, 25]. The optimal temperatures of keratinolytic proteases from other *Baciilus* were between 50 and 60°C [21, 24]. According to pH and thermal stability of both purified keratinase I and II, they were similar with those obtained from other species [8, 10, 29].

6. Effects of Metal Ions, Reducing Agents and Inhibitors

Keratinase I was moderately inhibited by Ca^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}, TLCK and EDTA, and highly inhibited by Cu^{2+}, Fe^{2+}, Hg^{2+}, Zn^{2+}, Fe^{3+}, NEM and *p*CMB. It was activated by Li^{+}, DTT, GSH and β-Me (Table 3). Keratinase II was moderately inhibited by Cd^{2+}, Co^{2+}, Fe^{2+}, Ni^{2+}, EDTA, NEM, *p*CMB and TLCK, and highly inhibited by Cu^{2+}, Hg^{2+}, Zn^{2+}, Fe^{3+} and PMSF. However, it was activated by DTT, GSH and β-Me (Table 3).

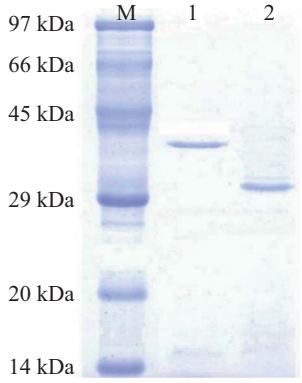

Fig. 5. SDS-PAGE of keratinase I and II (After electrophoresis, the proteins were stained with Coomassie blue R-250. Lane 1: keratinase I; Lane 2: keratinase II; Lane M: Low molecular weight proteins marker, 14~97 kDa).

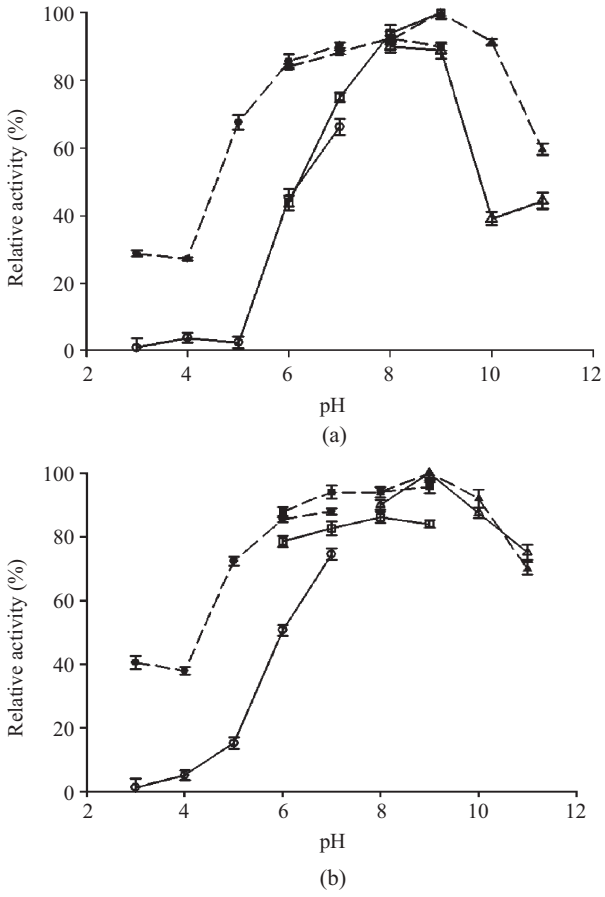

Fig. 6. Effect of pH on the activity of purified keratinase I (a) and II (b) (– – –, stability; ——, optimal pH; ● and ○, pH 3.0-7.0 in 20 mM citrate buffer; ■ and □, pH 6.0-9.0 in 20 mM sodium phosphate buffer; ▲ and Δ, pH 8.0-11.0 in 20 mM sodium carbonate buffer).

Many studies have used the reducing agents such as DTT, β-ME and reduced glutathione to enhance keratinase activity [3, 5, 19]. They also observed that reducing agents can decrease the disulfide bonds of keratin by sulfitolysis, and also make keratinase much easier access to substrate for keratinolysis [6, 14]. In other cases, transition and heavy metals including Hg^{2+} [19, 25], Zn^{2+} [6, 25], Cu^{2+} [16, 25] and Co^{2+} [6] inhibited keratinase activity.

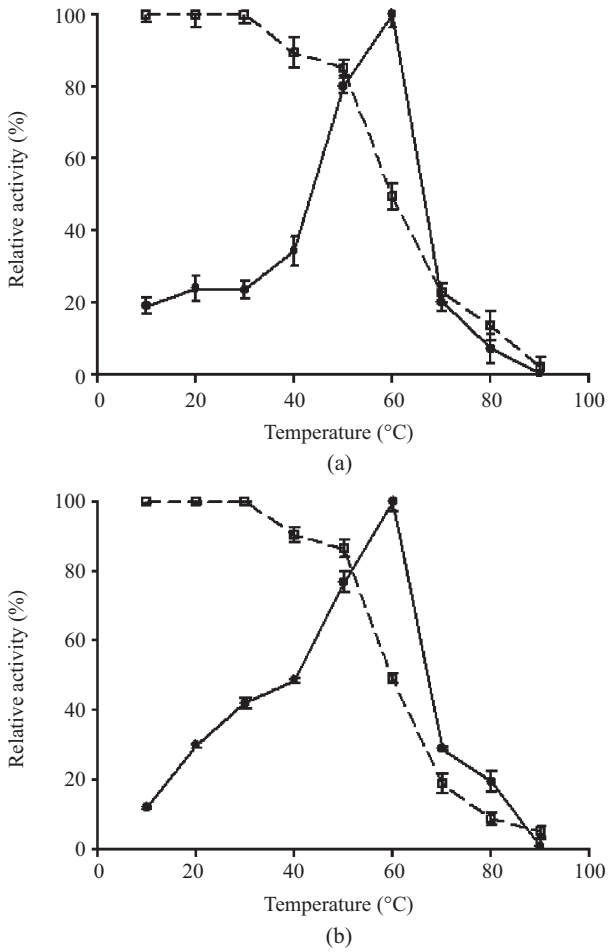

Fig. 7. Effect of temperature on the activity of purified keratinase I (a) and II (b) (—●—, Optimal temperature; – □ –, thermal stability).

Gupta and Ramnani [10] considered that most of keratinases belong to subtilisin group of proteases, because they possess serine or metallo catalytic center and have pH optima from neutral to alkaline. However, few keratinases were cysteine protease [18, 20, 21]. Rozs *et al*. [21] observed that a novel thiol protease from *Bacillus licheniformis* strain could be inhibited by *p*CMB and Hg^{2+}. According to the data obtained in this study, the purified keratinase I (sensitivity to NEM and *p*CMB, and moderately inhibited by metals such as Hg^{2+} and Zn^{2+}) and keratinase II (highly inhibited by Cu^{2+}, Hg^{2+}, Zn^{2+}, Fe^{3+} and PMSF) were considered to be cysteine and serine proteases, respectively.

In summary, the isolated *Bacillus licheniformis* YJ4, with high keratinolytic activity, could use the natural wastes, feather and rice husk, as substrates, and consequently lowered the cost for the production of keratinase. The produced kerati-

Table 3. Effect of metal ions and chemicals on the activity of purified keratinase I and II.

Metals	Relative Activity (%)					
	Keratinase I			Keratinase II		
	Concentration (mM)			Concentration (mM)		
	1.0	5.0	10.0	1.0	5.0	10.0
None	100.0	100.0	100.0	100.0	100.0	100.0
Li^{+}	115.3 ± 2.9	120.2 ± 0.8	102.0 ± 1.6	107.1 ± 1.1	101.2 ± 0.6	90.8 ± 1.2
Ba^{2+}	95.8 ± 0.7	102.9 ± 2.4	90.2 ± 0.1	102.9 ± 1.4	102.1 ± 1.3	71.9 ± 2.4
Ca^{2+}	87.3 ± 2.4	88.5 ± 0.1	78.4 ± 1.6	101.5 ± 0.3	102.4 ± 1.2	80.5 ± 1.8
Cd^{2+}	105.1 ± 3.5	95.2 ± 1.9	70.6 ± 2.0	91.0 ± 0.1	85.1 ± 2.4	68.7 ± 1,8
Co^{2+}	75.4 ± 2.1	84.6 ± 0.2	92.2 ± 0.2	86.2 ± 1.1	83.9 ± 0.3	61.3 ± 1.0
Cu^{2+}	71.2 ± 1.4	68.3 ± 2.4	10.8 ± 0.8	77.2 ± 2.4	55.7 ± 0.7	34.6 ± 0.7
Fe^{2+}	72.9 ± 1.4	59.6 ± 0.1	2.0 ± 1.6	88.1 ± 1.4	82.1 ± 0.6	50.8 ± 2.7
Hg^{2+}	92.4 ± 2.1	48.1 ± 2.7	39.2 ± 2.4	36.5 ± 0.3	5.4 ± 1.8	1.0 ± 0.3
Ni^{2+}	73.7 ± 0.7	73.1 ± 3.1	64.7 ± 0.1	80.1 ± 1.3	80.4 ± 0.3	58.7 ± 1.6
Zn^{2+}	28.0 ± 3.5	2.8 ± 0.8	0.0 ± 2.4	104.5 ± 1.0	27.1 ± 0.4	3.9 ± 0.4
Fe^{3+}	0.0 ± 2.8	0.0 ± 2.4	0.0 ± 1.6	101.0 ± 1.1	26.8 ± 0.3	1.0 ± 0.1
Chemicals	Concentration (mM)			Concentration (mM)		
	1.0	2.0	5.0	1.0	2.0	5.0
DTT	143.4 ± 4.6	126.7 ± .07	133.0 ± 1.3	104.8 ± 2.3	100.5 ± 0.1	114.8 ± 3.5
EDTA	94.3 ± 0.1	76.7 ± .07	72.3 ± 0.5	87.3 ± 2.6	83.2 ± 0.2	88.3 ± 1.8
GSH	134.0 ± 4.1	117.2 ± 2.8	121.3 ± 4.7	103.2 ± 2.5	102.9 ± 1.3	113.0 ± 0.1
NEM	79.3 ± 1.5	75.0 ± 3.5	45.7 ± 4.3	86.4 ± 3.3	87.2 ± 0.2	67.0 ± 3.5
β-ME	115.1 ± 1.5	120.7 ± 4.2	134.0 ± 1.7	89.6 ± 3.8	107.5 ± 1.3	115.2 ± 1.8
*p*CMB	77.7 ± 0.8	72.6 ± 0.8	50.0 ± 4.8	94.6 ± 0.8	91.5 ± 0.9	79.1 ± 0.2
PMSF	106.6 ± 3.8	104.3 ± 0.7	104.3 ± 1.7	33.2 ± 2.8	38.8 ± 0.9	18.7 ± 0.4
TLCK	74.5 ± 2.3	77.6 ± 0.5	83.0 ± 4.8	74.4 ± 1.3	79.5 ± 3.4	68.7 ± 1.4

nase I and II were considered to be cysteine and serine proteases, respectively.

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