Appl Biochem Biotechnol (2010) 162:780–794
DOI 10.1007/s12010-009-8802-x

Keratinase Production by Endophytic *Penicillium* spp. Morsy1 Under Solid-State Fermentation Using Rice Straw

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Received: 10 July 2009 / Accepted: 28 September 2009 /
Published online: 14 October 2009

Abstract Among all endophytic keratinolytic fungal isolates recovered from marine soft coral *Dendronephthya hemprichii*, *Penicillium* spp. Morsy1 was selected as the hyperactive keratinolytic strain under solid substrate fermentation of different agriculture and poultry wastes. The optimization of extraction process, physicochemical parameters affecting the keratinase production in solid-state fermentation, and the purified keratinase parameters were studied. Maximum keratinase activity (1,600 U g^{-1}, initial dry substrate) was recovered from moldy bran with 0.1% Tween 80. The optimized production conditions were rice straw as carbon source, pH of medium 6, growth temperature 26 °C, initial moisture content of 80% (*v*/*w*), inoculum size of 10^5 spores ml^{-1}, and an average particle size of the substrate 0.6 mm (3,560 U g^{-1}, initial dry substrate after 5 days of fermentation). Two types of keratinase (Ahm1 and Ahm2) were purified from the culture supernatant through ammonium sulfate precipitation, DEAE-Sepharose, and gel filtration chromatography. Enzyme molecular weights were 19 kDa (Ahm1) and 40 kDa (Ahm2). The kinetic parameters of purified keratinases were optimized for the hydrolysis of azokeratin by Ahm1 (pH 7.0–8.0, stable in pH range of 6.0 to 8.0 at 50 °C) and Ahm2 enzymes (pH 10.0–11.0, stable in pH range of 6.0 to 11.0 at 60–65 °C). Whereas inhibitors of serine (phenylmethylsulfonyl fluoride) and cysteine (iodoacetamide) proteases had minor effects on both Ahm1 and Ahm2 activity, both keratinases were strongly inhibited by chelating agents EDTA and EGTA. These findings suggest that serine and cysteine residues are not involved in the catalytic mechanisms, and they are metalloproteases.

Keywords Endophytic *Penicillium* spp. · Keratinase · Solid-state fermentation · Process parameters · Purification

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Introduction

Microbial keratinase (EC 3.4.99.11), one of a group of proteinase enzymes, has several biotechnological applications, such as hydrolyze keratinous wastes as feather, hair, and horn to clear obstructions in the sewage system during wastewater treatment and in food industry; detergent formulations for eliminating horny epithelial cells adhering to textile fibers; ecologically friendly leather industry; and pharmaceutical and cosmetic industries. Alternatively, they can be used in the conversion of poultry or agroindustrial wastes into value products including rare amino acids (serine, cysteine, and proline), peptides, fertilizers, glues, films, foils, and low-cost keratin source for biotechnological applications and nutritional improvement of wastes for livestock feed. Solid-state fermentation (SSF) has many advantages over submerged fermentation (SMF) in producing products for the food, feed, pharmaceutical, and agricultural industries, especially for fungal cultures [1–3]. These include high volumetric productivity, relatively high concentration of product, generation of less effluent, simple fermentation equipment, and purification procedures, which are less costly. Additionally, the advantages of using low-cost natural material in SSF processes, which greatly reduces pollution problems, are of special economic interest for the countries with abundance of biomass, agroindustrial, and poultry residues. Reducing the costs of protease production by optimizing the fermentation medium and the process is the goal of basic research for industrial applications. A number of poultry and agroindustrial residues have been exploited to analyze their potential to be used as substrates for protease production in SSF, and then protease produced by SSF process has greater economic feasibility. Among the various groups of microorganisms used in SSF, filamentous fungi are most widely exploited in the practical production of a wide range of extracellular enzyme or chemicals due to their ability to grow on complex solid substrates and hyperproduction of enzymes [1, 2, 4–6]. There are no reports in the current literature dealing with the production of keratinase by endophytic fungi or any other kind of endophytic microorganism using poultry or agroindustrial residues. In the present study, we report the optimization of extraction process, physicochemical parameters for the production of keratinolytic enzymes by endophytic *Penicillium* spp. Morsy1 under SSF, as well as the purification and characterization of *Penicillium* spp. Morsy1 keratinase.

Materials and Methods

The Collection of Coral Samples, Isolation, and Identification of Endopytic Fungal Strains

The collection of coral samples and isolation of endopytic fungal strains from the soft coral *Dendronephthya hemprichii* was previously described [7]. Keratinolytic isolate, showing potent keratinase activity upon a large number of different agricultural and poultry wastes, was grown on Czapek's agar with yeast extract, malt extract agar, and glycerol agar as described by Pitt [8]; then morphological and physiological observations were made on cultures that had been grown 7 days at 5, 25, and 37 °C in the dark [8, 9]. All fungal strains were maintained on potato dextrose agar (PDA) and modified potato carrot agar slants [10].

Proteolytic and Keratinolytic Activity

Milk agar plates (5 g L^{-1} peptone, 3 g L^{-1} yeast extract, 100 ml L^{-1} sterile skimmed milk, and 15 g L^{-1} agar) were prepared for primary screening of proteolytic activity. Fungal

isolates were inoculated onto plates and incubated at 28 °C for 7 days. Strains producing clearing zones in this medium were selected for keratinolytic activity. Keratinase activity was determined using azokeratin hydrolysis method [11]. Enzyme activity was expressed as units per gram of initial dry substrate (U g^{-1} IDS) in the solid-state experiments and unit per milliliter (U ml^{-1}) in the SMF.

Inoculum Preparation

Fungi were grown on PDA slants at 28 °C for 5 days. The spores were harvested with sterile distilled water containing 0.1% Tween 80 and used as inoculum after adjusting the desired spore count (10^5 spores g^{-1}), and 2% of spore suspension containing 10^5 spores ml^{-1} was used for SMF inoculation.

Growth Condition

Submerged Fermentation

Fermentation was carried out by seeding inoculum (2%, *v*/*v*) in Erlenmeyer flasks (250 ml) containing 50 ml of the mineral medium (grams per liter) 0.3; $(NH4)_2SO_4$, 0.5; NaCl, 0.5; KH_2PO_4, 0.4; $CaCl_2$ supplemented with 1% (*w*/*v*) rice straw. After incubation at 30 °C under agitation (200 rpm) for 5 days, the clear supernatant was obtained by centrifugation at 5,000 rpm at 4 °C for 15 min. The clear supernatant was used for determination of enzyme activity and protein content.

Solid-State Fermentation

To evaluate the keratinase activity of the proteolytic isolates under SSF upon different wastes, 250-ml Erlenmeyer flasks containing 10 g of one of agriculture or poultry waste separately, barley straw, wheat straw, rice straw, or corn cob (agriculture wastes), raw feathers, bovine hair, horn, or nails (poultry wastes), or wool (dry basis) and moisture content adjusted to 60% (*w*/*v*) by adequate distilled water containing (grams) 0.3; $(NH4)_2SO_4$, 0.5; NaCl, 0.5; KH_2PO_4, 0.4; $CaCl_2$ were seeded with 10^5 spores g^{-1} of each isolate separately. Fermentations were carried out for 7 days at 30 °C. Keratinolytic enzymes were extracted from the fermented solid.

Enzyme Extraction

After incubation, 3 g of the moldy bran was extracted with various extractants such as distilled water (control), Tween 80 (0.1%), NaCl (1.0%), ethanol 10% + glycerol 3%, citrate phosphate buffer (pH 5.0, 0.2 M), and phosphate buffer (pH 7.0, 0.2 M) separately on a rotary shaker at 150 rpm for 60 min and filtered under vacuum. The culture filtrate was used as an enzyme extract for assay of enzyme activity and protein content [2, 12].

Optimization of Keratinase Production Parameters

The keratinase production parameters were optimized under SSF, and the parameters optimized earlier were incorporated in subsequent experiments. The sequence of parameters that were standardized was time course of keratinase production (1–10 days) in the presence of rice straw as a substrate at 30 °C and pH 7, 0.5 mm particle size, and 100% moisture

contents, pH (different initial pH values 4, 5, 6, 7, and 8 were used), temperature of the fermentation (20, 22, 24, 26, 28, 30, 32, and 34°C), inoculum size (log of spore concentration per gram ranged from 1 to 10), initial moisture content (IMC; 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and100%), and particle size (0.2, 0.4, 0.6, 0.8, 1.0, 2.0, and >2.0 mm).

Enzyme Purification

The moldy bran was extracted as described before. The cell debris was removed by filtration under vacuum, and clear supernatant was lyophilized and used as cell-free extract for further purification by chromatography. The cell-free extract was dissolved in 50 mM sodium acetate buffer (pH 7.0), and proteins in the cell-free extract were concentrated by salting out with linear gradient of ammonium sulfate saturation and allow standing overnight. The precipitate formed was collected by centrifugation and then dissolved in 20 ml of 50 mM sodium acetate buffer, pH 7.0; the enzyme solution was dialyzed against the same buffer. The dialyzed solution was applied to DEAE-Sepharose CL-6B column (5 × 30 cm) that had been equilibrated and eluted with 50 mM sodium acetate buffer containing 0.5 M NaCl, at a flow rate of 30 ml h^{-1} (5 ml fractions were collected). The protease fractions were pooled, concentrated, dialyzed against the same buffer, and lyophilized. The lyophilized fraction from the previous step was dissolved in 5.0 ml of 50 mM sodium acetate buffer (pH 7.0) and loaded into a Sephacryl S-200 column chromatography (2.50 × 75 cm), equilibrated, and eluted with 50 mM sodium acetate buffer containing 0.5 M NaCl, at a flow rate of 15 ml h^{-1}. The resulting active fractions were collected and used as the purified keratinase preparations. All purification steps were done at 4°C.

Protein Assay and Electrophoresis

During purification, protein was estimated by measuring absorbance at 280 nm, with bovine serum albumin as the standard [13, 14]. Polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the purity and the molecular mass of the purified enzyme, according to the method of Laemmli [15]. The standard proteins (Sigma) used for calibration were phosphorylase (97.4 kDa), albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20.1 kDa), and α-lactabumin (14.4 kDa). Protein was stained with Coomassie brilliant blue R-250.

Characterization of Purified Keratinases

Optimization of purified keratinase parameters was obtained. Optimum pH was determined by assaying the keratinase activity at various pH using appropriate 0.25 M buffer systems (pH 3–6, citrate–phosphate; pH 7, phosphate; pH 8–9, Tris–HCl; and pH 10.0–12.0, glycine–NaOH buffer). Optimum temperature was determined by carrying out reaction at optimized pH with varying temperatures (20, 25, 30, 35, 40, 45, 50, 55, 60, 70, and 75°C).

The effect of 10 mM of oxidizing (H_2O_2) and reducing agents (β-mercaptoethanol) on enzyme activity was determined after preincubation at 30°C for 12 h. On the other hand, different detergents (Triton X-100, Tween-80, and sodium dodecyl sulfate (SDS)) at final concentration 2% and 5% were preincubated with enzyme at 30°C for 1 h. Purified keratinases were preincubated for 1 h with different metal ions including Ca^{+2}, Mn^{+2}, Mg^{+2}, Co^{+2}, Cu^{+2}, Fe^{+2}, Hg^{+2}, Cd^{+2}, and Pb^{+2} at a final concentration of 5 mM. Moreover,

purified keratinases were preincubated for 30 min at 30°C with several types of protease inhibitors, 5 mM of phenylmethylsulfonyl fluoride (PMSF; serine protease inhibitor); 0.1 mM iodoacetamide (cysteine protease inhibitor); chelating agents, EDTA and EGTA, at final concentration of 10 and 50 mM (metallo protease inhibitors), separately. Residual activities in the presence of each chemical was obtained and compared with the control activity (without inhibitors as 100%).

Results and Discussion

Screening and Selection

Only eight endophytic fungal isolates showed pronounced clearing zones on skimmed milk agar plate. The keratinase activity of the selected proteolytic fungi upon different waste materials in SSF (Table 1) showed that endophytic marine fungi under the isolation number Morsy1was the hyperactive keratinase strain using rice straw as favored substrate for keratinase production (1,170.42 U g^{-1}), and then it was selected for optimization of various parameters under SSF conditions. Data in Table 1 indicated that Morsy1 keratinolytic enzymes were largely constitutive, requiring different substances as an exogenous inducer. However, Priest [16] reported that the microbial keratinolytic enzyme might be constitutive or partially inducible. The depression of keratinolytic protease in some fungi such as *Chrysosporium keratinophilum* and *Candida albicans* differentiates their exosecretion from constitutively expressed cell-bound kerantinolytic protease [17, 18]. Many authors used natural wastes such as feather as favored substrate for enumeration and keratinase production by microbial groups such as bacteria and fungi [3, 18, 19]. To our knowledge, this is the first report on the keratinase production by endophytic fugal strains of soft coral using rice straw.

Identification of Keratinolytic Morsy1 Strain

Keratinolytic Morsy1 strain was identified using the methodology of Pitt [8, 9]. Fungal conidiophore and conidia structures detected under scanning electron micrographs were typical of the genus *Penicillium* [8, 9] (Fig. 1a, b).

Table 1 Keratinase activity (units per gram IDS) of endophytic proteolytic fungal isolates after 5 days of incubation at 30°C in medium containing 10 g L^{-1} of either natural wastes.

Isolate	Substrate								
	Barley straw	Wheat straw	Rice straw	Corn cob	Raw feather	Bovine hair	Nail	Wool	Horn
Morsy1	908.74	1,012.00	1,170.42	869.80	1,100.12	333.80	730.93	918.80	1,040.80
Morsy2	432.11	844.91	950.89	792.52	1,048.52	739.52	439.42	319.52	420.56
Morsy3	246.17	122.71	131.90	214.32	121.35	412.43	112.00	421.30	114.30
Morsy4	14.15	47.46	48.65	125.30	125.30	15.30	15.30	12.30	25.30
Morsy5	212.89	129.09	228.00	330.86	1,000.12	1,130.00	634.82	590.12	1,008.00
Morsy6	704.00	855.85	990.00	720.88	880.00	900.08	470.02	660.22	820.43
Morsy7	214.00	150.90	420.00	160.86	250.60	440.50	118.00	648.66	566.14
Morsy8	19.80	5.62	11.44	16.60	180.64	148.60	158.00	36.60	51.22

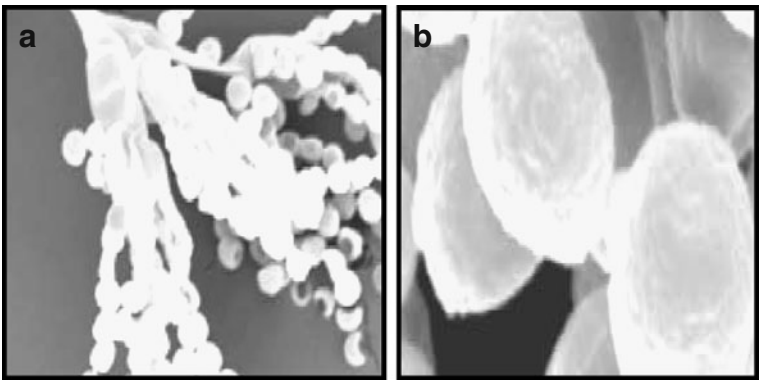

Fig. 1 Scanning electron micrographs of *Penicilium* spp. Morsy1 **a** conidiophores and **b** conidia

Optimization of Keratinase Extraction Process

Various extractants used for keratinase extraction from moldy bran of *Penicillium* spp. Morsy1. 0.1% of Tween 80 proved to be suitable for efficient recovery of the enzyme (1,600 U g^{-1}, Table 2) compared to 970 U g^{-1} of enzyme activity extracted by water. This observation is in disagreement with the maximum proteases extracted from moldy bran of *Rhizopus oligosporus* [4], *Mucor bacilliformis* [2] with 1.0% sodium chloride solution, and moldy bran of *Rhizopus oryzae* with a mixture of 10% ethanol with 3% glycerol [20]. Location of enzyme, hydrophobic/hydrophilic nature of fungal mycelia, ionic/hydrogen bonds, and van der Waal forces appear to determine the efficiency of the extractant as pointed out by Fernandez-Lahore et al. [2].

Optimization of Production Parameters

Production of Protease in SMF and SSF

Results in Table 3 supported that *Penicillium* spp. Morsy1was able to produce keratinase using rice straw as substrate in both SMF and SSF. SSF has gained numerous advantages for the production of high titers of keratinase enzymes (relative activity was 51.73% and 100% using SSF in Petri dish and Erlenmeyer flasks, respectively) rather than submerged fermentation (relative activity was 45.47% and 79.11% using shaking flasks and fermentor

Table 2 Effect of enzyme extractants on keratinase activity of *Penicillium* sp. Morsy1 grown under SSF conditions.

Extractant	Keratinase activity (U g^{-1} IDS)
Distilled water (control)	970
NaCl (1.0%)	1,462
Ethanol (10%) + glycerol (3%)	987
Tween 80 (0.1%)	1,600
Citrate–phosphate buffer (pH 5.0, 0.2 M)	743
Phosphate buffer (pH 7.0, 0.2 M)	1,008

Table 3 Comparison of relative Morsy1 keratinase activity in different fermentation process.

Fermentation process	Relative activity (%)
Solid-state fermentation in Petri dish	51.73
Solid-state fermentation in 250 ml Erlenmeyer flask	100[a]
Submerged fermentation in 250 ml, shake flask	45.47
Submerged fermentation in 10 L fermentor	79.11

[a] 100%=1,600.24 U g^{-1} IDS

10 L, respectively). Thus, SSF can be used as alternative economical cultivation system for the production of high-cost industrial enzymes as proteases by different fungi [1–6].

Typical Time Course of Enzyme Production

Different incubation periods (1–10 days) were employed to study their effect on keratinase production. The fermentation was carried out at 30 °C and pH 7. Keratinolytic activity was not detected during the first 24 h; thereafter, the enzyme levels increased, reaching a maximum after 5 days (1,834.20 U g^{-1}), and it gradually reduced with longer incubation times (Fig. 2) due to increasing of pH of fermentation medium to pH 11 (data not shown). The optimum incubation period achieved by this step was fixed in the subsequent experiments

Effect of pH and Temperature

Penicillium spp. Morsy1 yielded maximum keratinase production (2,010.80 U g^{-1}) at pH 6.0 (Fig. 3a) and declined, thereafter, with the initial medium alkalinity. This observation supports the finding that fungal extracellular enzymes are produced in high titer with pH optima at the growth pH [21].

The correlation between enzyme production and incubation temperature was shown in Fig. 3b. Incubation temperature of 26 °C resulted in maximal enzyme production by

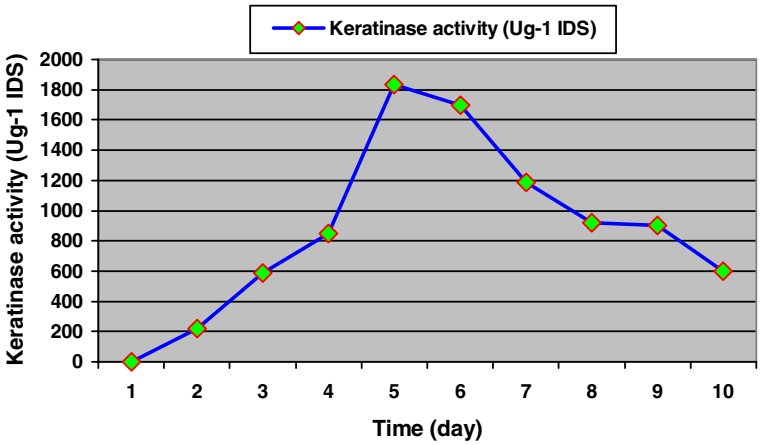

Fig. 2 Typical time course of keratinase production by *Penicillium* spp. Morsy1 in SSF conditions

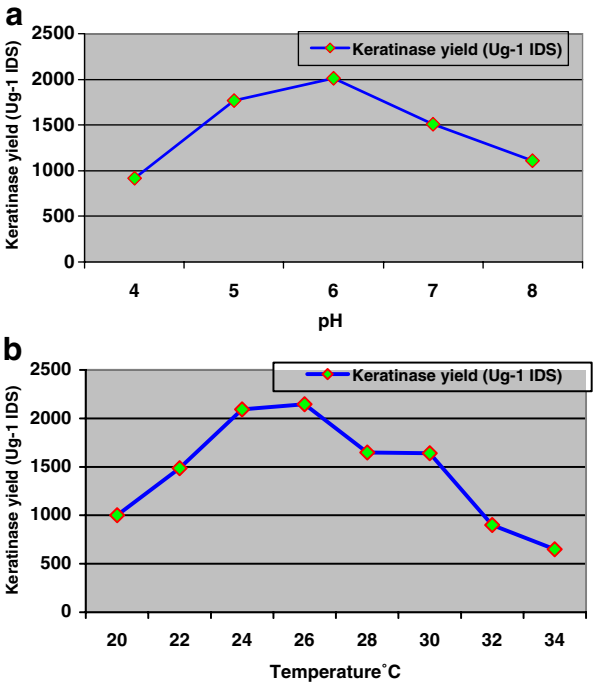

Fig. 3 **a** Effect of pH on keratinase production by *Penicillium* spp. Morsy1 in SSF. **b** Effect of temperature on keratinase production by *Penicillium* spp. Morsy1 in SSF

Morsy1 (2,144.90 $U g^{-1}$). Incubation temperature is characteristic of an organism and profoundly affects the enzyme yield and duration of enzyme synthesis phase [12]. These findings are in line with those obtained for keratinase production by *Penicillium* spp., *Bacillus subtilis*, and poultry farm soil fungi [1, 3, 22].

Inoculum Size

Inoculum size is a very important factor affecting cell growth and product formation. The pattern of keratinase production with respect to inoculum size indicated that, with increase in inoculum size, Morsy1 strain showed maximum keratinase yield (2,480 U g^{-1}) at a concentration of 10^5 spore g^{-1} IDS, and enzyme activity sharply decreased when inoculum sizes were out of this range (Table 4). Larger inoculum size has been shown to affect adversely the yield of keratinase by *Beauveria bassiana* [12].

Initial Moisture Level

With respect to the requirement of IMC for keratinase production, Morsy1 exhibited maximum keratinase yield (2,805.63 U g^{-1}) at 80% initial moisture content (Fig. 4). Results and visual observation indicated the critical importance of moisture content of medium. A lower moisture level led to dry culture and sparse growth with subsequent lower production of enzymes, while high moisture level decreased porosity, enhanced formation of liquid mycelium, and created an additional barrier for growth diffusion into the substrate. Water

Table 4 Effect of inoculum size on keratinase production by *Penicillium* sp. Morsy1 in SSF.

Log of spore concentration/ml	Keratinase yield (U g^{-1} IDS)
2	650
3	1,008
4	1,827
5	2,480
6	1,940
7	1,625
8	1,101
9	823
10	600

used in SSF has profound effects on the physicochemical properties of solids and growth of the producing organism, which affects process of hydrolytic enzyme productivities under SSF conditions [3] by *Penicillium* spp. [1], other filamentous fungi [5], and *Bacillus licheniformis* [23].

Substrate Particle Size

Particle size of rice straw and, therefore, the specific surface area showed a significant effect on the hyperproduction of keratinase by *Penicillium* spp. Morsy1 (Table 5). Maximum keratinase productivity (3,560 U g^{-1}) was obtained from 0.6-mm-sized particles and was lower with bigger or smaller particles. These results are in line with that obtained for particle size of sorghum straw for xylanase production by *Thermomyces lanuginosus* under SSF [24]. On the other hand, the substrate particle size did not significantly affect the enzyme production by *Aspergillus awamori* in SSF [25]. Lesser enzyme titers obtained on smaller substrate particle size may be attributed to increased mycelial thickness around the solid substrate particles that resulted in decreased porosity of the substrate bed and diffusivity of oxygen; then, the filamentous fungus could not penetrate deep into the pores

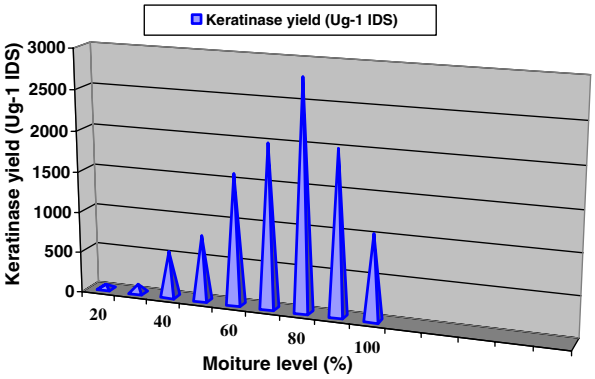

Fig. 4 Effect of initial moisture level on keratinase production by *Penicillium* spp. Morsy1 in SSF

Table 5 Effect of particle size on keratinase production by *Penicillium* sp. Morsy1 in SSF.

Particle size (mm)	Keratinase yield (U g^{-1} IDS)
0.2	1,014
0.4	2,129
0.6	3,560
0.8	3,120
1.0	2,648
2.0	2,184
>2.0	1,408

and, hence, into the substrate particles. The saturated surface area for growth with larger particle sizes was less, and productivity was correspondingly less [26]. Rice straw with 0.6-mm particle size possibly provided sufficient surface area and aeration to fungus for growth resulting in increased keratinase production.

Purification of Morsy1 Keratinases

Keratinase was purified from the culture filtrate by 80% saturation of ammonium sulfate (the fraction showed the highest keratinase activity) followed by DEAE-Sepharose and Sephacryl S-200 column chromatography. The purification steps from the crude extract of *Penicillium* spp. Morsy1 are summarized in Table 6. As shown in Fig. 5, two keratinolytic proteins peaks were separated and designated as Ahm1 and Ahm2. Both of these keratinases were purified by successive Sephacryl S-200 gel chromatography to homogeneity as judged by SDS-PAGE (Fig. 6). The purification procedure yielded a 3.56-fold purified Ahm1 with 4. 93% recovery and 17.47-fold purified Ahm2 with 26.67% recovery. The final specific activity of Ahm1 and Ahm2 were 8.06 and 39.593 U mg^{-1} of protein, respectively. The molecular weight of Ahm1 and Ahm2 were estimated to be 19 and 40 kDa (Fig. 6). Microbial proteases that had the similar molecular weight as Morsy1 keratinase Ahm1 included the metalloproteases of *Pseudomonas aeruginosa* (18 kDa) [27] and *Aeromonas caviae* (19 kDa) [28]. The microbial proteases that had the similar molecular weight of Ahm2 keratinase were metalloproteases of *Bacillus cereus* (38 kDa) [29] and *Bacillus* sp. PS719 (42 kDa) [30].

Table 6 Purification of Ahm1 and Ahm2 keratinases.

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification folds
Crude extract	662	1,500	2.27	100	1
80% $(NH_4)_2$ SO_4	300	1,360	4.53	90.68	2
DEAE-Sepharose (Ahm1)	20	120	6.02	8.00	2.66
Sephacryl S-200 (Ahm1)	9	74	8.06	4.93	3.56
DEAE-Sepharose (Ahm2)	60	820	13.47	54.67	5.90
Sephacryl S-200 (Ahm2)	10	400	39.59	26.67	17.47

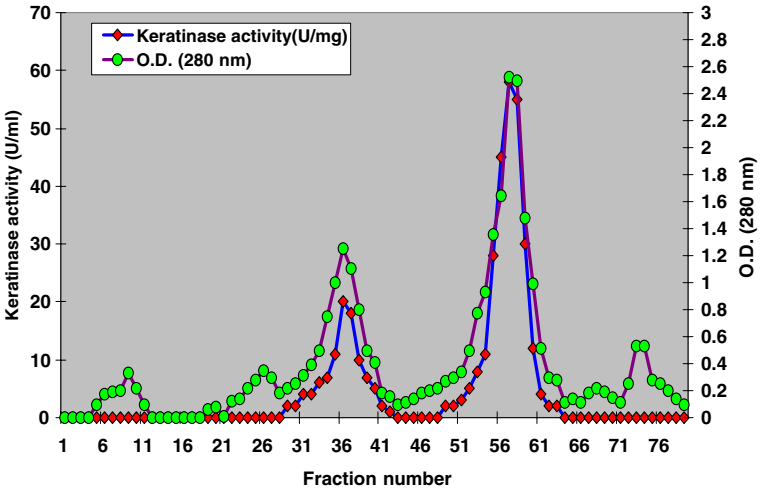

Fig. 5 Elution profile of Morsy1 keratinases on DEAE-Sepharose CL-6B column chromatography

Optimization of Purified Keratinase Parameters

The purified enzymes Ahm1 and Ahm2 of Morsy1 strain grown under SSF condition exhibited maximum keratinase activity at pH 7.0–8.0 and 10.0–11.0, respectively (Fig. 7a, b). Below or above these levels, the activity decreased gradually. Ahm1 was stable in the pH range of 6.0 to 8.0, but Ahm2 was stable in the pH range of 6.0 to 11.0 (Fig. 7a, b). These data are in agreement with those obtained for thermostable alkaline keratinolytic enzymes from *C. keratinophilum* [17] and *Penicillium* spp. under SSF conditions [1]. On the other hand, the enzyme produced by *M. bacilliformis* [2] and *R. oligosporus* [4] showed maximum activity in the acidic range.

Moreover, a classical pattern of temperature–activity relationship with temperature optima at 50 and 60–65 °C was observed with Ahm1 and Ahm2, respectively (Fig. 8a, b).

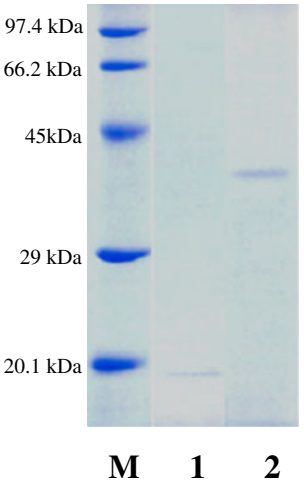

Fig. 6 SDS-PAGE of the purified Morsy1 keratinases. *Lane M* standard molecular mass markers, *lane 1* Ahm1 keratinase, *lane 2* Ahm2 keratinase; proteins were stained with Coomassie brilliant blue R-250

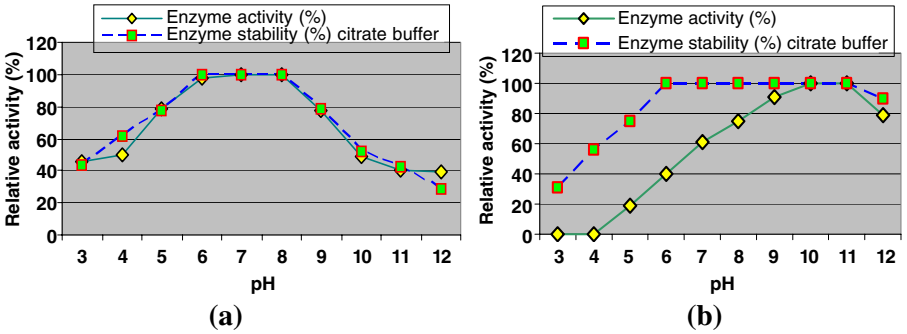

Fig. 7 Effect of pH on the activity and stability of keratinase Ahm1 (**a**) and Ahm2 (**b**)

Whereas Ahm1 was stable at a temperature lower than 75 °C, Ahm2 was stable at temperature lower than 60 °C. Many investigators reported optimum reaction temperatures of 40–70 °C for keratinase activity and stability from other fungi, *Penicillium* spp., *C. keratinophilum*, *Penicillium janthinellum* CRC 87M-115 [1, 17, 31], and from *Bacillus* spp. [22, 29]. Thermostability of Ahm1 keratinase seems to be very useful in biotechnological application, which needs high temperatures as leather industry as reported for *C. keratinophilum* [17] and *Penicillium* spp. under SSF conditions [1].

Effects of Metal Ions and Some Inhibitors

Both purified keratinases were not somewhat inhibited by the presence of surfactants, oxidizing agent H_2O_2, or reducing agent β-mercaptoethanol (Table 7). The inhibitors of serine (PMSF) and cysteine (iodoacetamide) proteases had the minor effects on both Ahm1 and Ahm2 keratinase activity, which suggests that serine and cysteine residues are not involved in the catalytic mechanisms. In contrast, both keratinases were strongly inhibited by chelating agents EDTA and EGTA. Whereas EDTA at 10 and 50 mM inhibited 75% and 81% of Ahm1 as well as 22% and 89% of Ahm2 enzyme, EGTA at 10 and 50 mM inhibited 84% and 91% of Ahm1 and 55% and 78% of Ahm2, thus, suggesting that both Ahm1 and Ahm2 enzymes are metalloproteases.

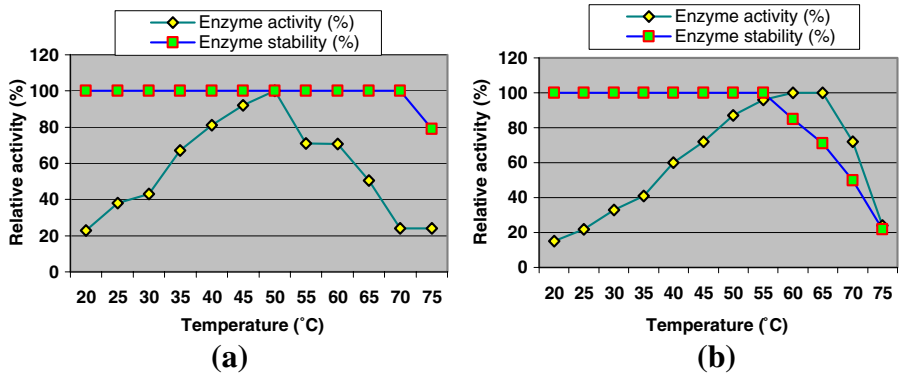

Fig. 8 Effect of temperature on the activity and stability of keratinase Ahm1 (**a**) and Ahm2 (**b**)

Table 7 Effect of metal ions and some enzyme inhibitors on Ahm1 and Ahm2 keratinases.

Chemical	Concentration	Residual activity (%)a	
		Ahm1	Ahm2
Control	0	100	100
Detergent (%)			
Triton X-100	2	98	100
	5	94	100
Tween-80	2	100	99
	5	100	94
SDS	2	100	88
	5	100	75
Oxidizing and reducing agent (mM)			
H_2O_2	10	97	100
β-Mercabtoethanol	10	97	98
Protease inhibitors (mM)			
PMSF	5	94	96
Iodoacetamide	0.1	92	94
EDTA	10	25	78
	50	19	11
EGTA	10	16	45
	50	9	22
Metal ions (mM)			
Mn^{2+}	5	400	320
Cu^{2+}	5	44	112
Mg^{2+}	5	130	210
Ca^{2}	5	190	360
Fe^{3+}	5	90	75
Pb^{2}	5	55	0
Cd^{2+}	5	106	90
Co^{2+}	5	105	60
Hg^{2+}	5	70	22
Organic solvents (%)			
DMSO	5	100	120
Acetone	5	100	113

[a] The activity of keratinase without any reagent was defined as 100%

The purified keratinase Ahm1 showed more strong stability than Ahm2 in the presence of metals such as Fe^{3+}, Pb^{2}, and Hg^{+2} (Table 7). The presence of 5 mM of ions Mn^{+2}, Ca^{+2}, and Mg^{+2} increased the relative activity of Ahm1 up to 400%, 190%, and 130% and increased Ahm2 activity by 320%, 360%, and 210%, respectively, of the control. In the presence of an organic solvent, dimethyl sulfoxide (DMSO), and acetone, there was an increase in the activity of Ahm2 by 120% and 113% of the control (Table 7). Most of the keratinases found to date have been reported to be serine proteases, and a few are metalloproteases [32, 33]. Keratinolytic metalloproteases may have great biotechnological promise; they may overcome the limited proteolysis on the surface of insoluble keratin

particles because of restricted enzyme–substrate interaction as well as the metalloenzyme nature which presents a potential method of enzyme immobilization because of a reduced autolysis [32]. Ca^{2+}, Mg^{2+}, and Mn^{2+} have been reported to cause a 3-fold increase of the enzymatic activity [34]. These metal ions might be associated with the stabilization of the tertiary structure conformation of metalloproteases and protect these enzymes against thermal denaturation and autoproteolysis [35, 36].

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