Türk Biyokimya Dergisi [Turkish Journal of Biochemistry–Turk J Biochem] 2013; 38 (1) ; 68–74
doi: 10.5505/tjb.2013.37928

Research Article [Araştırma Makalesi]

Yayın tarihi 15 Nisan, 2013 © TurkJBiochem.com

[Published online 15 April, 2013]

Purification and properties of a novel fungal alkaline keratinase from *Cunninghamella echinulata*

[Yeni fungal alkali keratinazın *Cunninghamella echinulata*'dan saflaştırılması ve özelliklerinin tanımlanması]*

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Registered: 5 October 2012; Accepted: 21 January 2013

[Kayıt Tarihi : 05 Ekim 2012; Kabul Tarihi : 21 Ocak 2013]

ABSTRACT

Aim: The purification and characterization of an alkaline keratinase from *Cunninghamella echinulata.*

Materials and Methods: Feather-meal medium was used to isolate and screen the fungi. Acetone precipitation and lectin agarose affinity column were used in the purification. The effect of pH, temperature, metal ions and group modifying chemicals was tested.

Results: The purified keratinase is a serine protease with a molecular mass of 33kDa with an optimal pH of 4.5 and 10.0, and an optimum temperature of 30°C and 60°C. A 13.2-fold purification was obtained with affinity methods.

Discussion: *C.echinulata* keratinase was inhibited by PMSF, thus it could be a serine protease. Enzyme was inhibited by group specific reagents like TLCK, IAA, NEM and NAI indicating that serine, cysteine, tyrosine and lysine play an important role in the catalytic activity. There was no effect of metal-chelating agent on enzyme activity indicating that the enzyme is not a metalloenzyme; however, it is a metal-activated enzyme as the activity was enhanced by Mn^{2}+. Inhibitory effects of group specific reagents indicated that the enzyme is a serine protease which does not have any divalent ion requirement. The enzyme isolated also has appreciable activity at two different temperatures and pH values making it a versatile organism for industrial applications.

Key words: Keratinase, keratin, lectin agarose affinity column

Conflict of Interests: There is no conflict in interest.

ÖZET

Amaç: Çalışmanın amacı *Cunninghamella echinulata*'dan alkali keratinazın saflaştırılması ve karakterizasyonudur.

Gereç ve Yöntemler: Mantarın izole edilmesinde ve taranmasında temel tüylü besiyeri kullanılmıştır. Saflaştırmada aseton çöktürmesi ve lektin agaroz affinite kolonu teknikleri uygulanmıştır. Sıcaklık, pH, metal iyonları ve grup modifiye edici kimyasalların etkisi araştırılmıştır.

Bulgular: Saflaştırılan keratinaz, molekül kütlesi 33 kDa, optimum pH'sı 4.5 ve 10, optimum sıcaklığı ise 30°C ve 60°C olan serin proteazdır. Keratinaz affinite teknikleri ile 13.2 kat saflaştırılmıştır.

Sonuç: *C.echinulata* keratinazının PMSF ile inhibe olması bu enzimin serin proteaz olabileceğini göstermektedir. Enzim TLCK, IAA, NEM ve NAI gibi grup özgül ajanlar ile inhibe olmakta ve bu durum serin, sistein, tirozin ve lizin amino asitlerinin katalitik aktivitede önemli rol oynadığını ortaya koymaktadır. Metal-kelatlayıcı ajanların enzim aktivitesi üzerine bir etkilerinin olmayışı keratinazın metalloenzim olmadığını gösterse de enzim aktivitesinin Mn^{2}+ ile artışı enzimin metal ile aktive olduğunu belirtmektedir. Grup özgül ajanların inhibitor etkisi enzimin herhangi bir divalan iyona gereksinim duymayan bir serin proteaz olduğunu göstermektedir. İzole edilen enzimin iki farklı sıcaklık ve pH noktasında aktiviteye sahip olması endüstriyel uygulamalarda değişken olmasını sağlamaktadır.

Anahtar Kelimeler: Keratinaz, keratin, lektin agaroz afinite kolonu

Çıkar Çatışması: Çıkar çatışması bulunmamaktadır.

Introduction

Keratinases are produced by a large number of bacteria, actinomycetes, and fungi, with the unique ability to act on "hard to degrade" keratins which are found in bulk in the environment in the form of feather, nail, hair, hoof and so forth [1,2]. By virtue of this ability, they are being exploited in various biotechnological applications in the sectors of ecologically friendly leather processing, nutritional improvement of waste feather for livestock feed, and production of protein hydrolysates from keratinous waste materials [1,3]. In addition to this, they are also considered as desirable detergent additives which could replace the traditional proteases for removal of keratinous soils that are often encountered in laundry, such as collar washings to remove scurf and for eliminating horny epithelial cells adhered to textile fibers [1,2]. To get significant importance as detergent additives, they should be robust in terms of their compatibility towards oxidizing agents, detergents, and surfactants and stable under high temperature and alkaline pH [1,4]. However, oxidation stability is not a commonly encountered characteristic among well-known detergent compatible proteases and is generally induced in them by site-directed mutagenesis and protein engineering [5]. Thus, keratinases with robust characteristics like oxidation stability, alkaline stability, detergent compatibility, and temperature tolerance can fetch a good market among detergent proteases.

Most of the keratinases reported to date have been found to be serine proteases [6-10], and a few metalloproteases have shown keratinolytic activity [11,12].

In the current study, the production of keratinase enzyme by *Cunninghamella echinulata* and some of its main biochemical and physiological properties were investigated. Strain *C.echinulata* shows great promise in finding potential applications in keratin hydrolysis and keratinase production.

Materials and Methods

Chemicals

Chicken feathers were collected from a local poultry processing factory. All chemicals used were of analytical grade and were purchased from Sigma (India).

Isolation and screening of fungi

Soil samples were obtained from poultry dump yards in Bangalore and were screened for keratinase-producing fungi as follows: 1g of the soil sample was suspended in distilled water. After soil-sedimentation, 0.1ml of the supernatant was used as inoculum for isolation and screening by spread plate method on minimal medium supplemented with feather as the sole carbon and nitrogen source (feather meal plate). The pH of the medium was adjusted to 10.0 in order to isolate alkaliphilic microorganisms.

Preparation of native chicken feather

Native chicken feathers (white leghorn feathers) were cut with scissors to small pieces of 1-3 cm long, then, washed several times with tap water. Defatting of feather pieces was done by soaking them in a mixture of chloroform:methanol (1:1 v/v) for 2 days followed by chloroform:acetone:methanol (4:1:3 v/v/v) for 2 days. The solvent was replaced every day. Finally, the feathers were washed several times with tap water to eliminate the solvent residual, dried for 3 days at 50°C and ground using an electrical blender. The ground keratin was used for further studies [13].

Fungal isolation, purification and maintenance

The feather meal medium used for isolation and growth of the feather-degrading fungi contained the following constituents (g L^{-1}): NaCl (0.5), KH_2PO_4 (0.7), K_2HPO_4 (1.4), $MgSO_4$ (0.1) and ground keratin (10). To prepare the solid medium 15-20 g agar were used. The pH was adjusted to 10.0. Spread plate technique was followed to isolate the fungi. After 7 days of incubation, colonies were subcultured until pure colonies were obtained. These were maintained on Martin's rose bengal agar medium at 4°C. The isolates were identified by Agharkar Research Institute, Pune.

Preparation of ground keratin

Keratinolytic activity was measured with soluble keratin (0.5%, w/v) as substrate. Soluble keratin was prepared from white chicken feathers by the method of Wawrzkiewicz [14]. Native chicken feathers (10g) in 500 ml of dimethyl sulfoxide (DMSO) were heated in a reflux condenser at 100°C for 2 h. Soluble keratin was then precipitated by the addition of cold acetone at −20°C for 2 h, followed by cooling centrifugation at 8050×g for 10 min. The resulting precipitate was washed twice with distilled water and dried at 40°C in a hot air oven.

Production of keratinase

The fungus was cultivated for 14 days in feather meal medium. Mycelium was removed by filtration, the filtrate was centrifuged at 10000 x g for 10 min, and the supernatant was used as a crude enzyme preparation [8,15,16].

Assay of keratinase activity

Enzyme activity was determined with keratin solution as the substrate. One gram of quantified precipitate was dissolved in 20 ml of 0.05 mol/L NaOH. The pH was adjusted to 8.0 with 0.1 mol/L Tris and 0.1 mol/L HCl and the solution was diluted to 200 ml with 0.05 mol/L Tris-HCl buffer (pH 8.0).

The keratinase activity was assayed as follows: 1.0 ml of crude enzyme was diluted in Tris-HCl buffer (0.05 mol/L, pH 8.0) and was incubated with 1 ml keratin solution at 50 °C in a water bath for 10 min, and the reac-

tion was stopped by adding 2.0 ml of 0.4 mol/L trichloroacetic acid (TCA) [17]. The centrifugation was carried out at 8000 rpm for 10 minutes and the supernatant was used for determination of released amino acids by a modified method [18] of Lowry *et al*. [19].

Determination of protein concentration

The concentration of protein in the enzyme solutions was assayed by the modified Lowry's method of protein estimation [18,19] using bovine serum albumin as the standard.

Purification of keratinase

Culture broth was chilled, centrifuged (10000 rpm for 10 min) to remove cells and debris and the supernatant was collected. Keratinase was precipitated by pre-chilled acetone (30-80%) fractionation [20]. The acetone was added to culture filtrate in 3:1 (acetone:filtrate) ratio and incubated for 60 minutes at -20°C. The contents were subjected to centrifugation at 15000 rpm for 15 minutes. The supernatant was discarded carefully and pellet was dissolved in Tris-acetate buffer (pH 7).

Affinity chromatography of this sample was carried out using lectin agarose column. Protein concentration and specific activity of purified sample were determined.

Growth optimization

Keratinase activity at different incubation periods: Keratinase activity of the isolated strain was measured in keratin meal broth medium for 28 days and activity was checked at regular intervals.

Effects of medium pH, temperature of incubation and substrate concentration on the production of keratinase: The basal feather medium was prepared in the pH range of 3-12. The fungus was grown at the temperature range from 25°C to 65 °C, substrate range from 0.5 to 2.5% in feather medium and enzyme activity was determined.

Molecular weight determination

In order to determine homogeneity and molecular weight, the partially purified enzyme sample and known molecular weight markers were subjected to electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed with 12% polyacrylamide gels as described by Laemmli [21], and the gels were silver stained. The molecular weight marker used contained phosphorylase b (97.4 kDa), serum albumin (66.2kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa).

The band was sliced out, extracted and centrifuged. The supernatant was tested for enzyme activity.

Effect of pH and temperature on keratinase activity

The keratinase activity was studied over the pH range of 2.5-12.0 using different buffers. The optimum temperature for keratinase activity was determined at different incubation temperatures between 0 and 100°C [13].

Effect of metal ions and group specific reagents

Effect of different metal ions on keratinase enzyme was analyzed. Effects of Fe^{2+}, Cu^{2+}, Zn^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Hg^{2+}, Ba^{2+}, Na^{+}, Ag^{2+}, K^{+}, Li^{+} were tested. Different group specific reagents, which were prepared in 25mM concentration, were added to the partially purified enzyme preparations and incubated for 15 min at room temperature before being tested for keratinase activity.

Results

Screening for feather degrading fungi

Three keratinase producing fungal species were isolated from different poultry dump yards on medium containing feather as sole carbon and nitrogen source. They were identified as *Aspergillus versicolor* gr, *Cunninghamella echinulata* (Tax 1) and *Aspergillus flavus* link by the Agharkar Research Institute, Pune. Of these three isolates *Cunninghamella echinulata* showed maximum growth and activity and was therefore used for further studies (Figure 1).

Optimization of growth conditions

Maximum enzyme activity was observed on day 14 (Figure 2). The broth was harvested on this day and cell-free extract was obtained by removing the cells through centrifugation. This extract was used for further studies on the enzyme. Maximum growth was seen in the flasks with the substrate concentration of 1%, at pH 10, and temperature 28°C.

Enzyme purification

The ammonium sulphate precipitation method of partial purification was not deemed successful as there was no keratinase activity in any of the fractions.

The enzyme was thus purified by acetone precipitation. The pellet obtained by acetone precipitation showed significant enzyme activity and so this method was continued to be used for the partial purification of the enzyme. Acetone precipitate was further purified by the affinity chromatography method as described earlier. The total activity, specific activity, fold purification and yield of each fraction were determined (Table 1). The enzyme purified by acetone precipitation and lectin agarose chromatography showed the recovery of 57.4% and 39.73% respectively. The corresponding fold purifications were seen to be 3.24 and 13.17, and specific activity was seen to increase from 8.58 to 35.30 U/mg.

Molecular weight determination

On observation, it was found that no clear bands were visible in the cell-free extract. This was possibly due to the dilute nature of the sample. Acetone precipitated sample, however, showed one clear band and a number of diffuse bands of different proteins. The band was found to have enzyme activity.

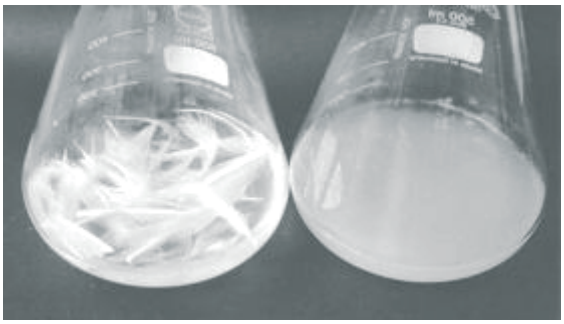

Figure 1. Feather degradation by *C.echinulata*

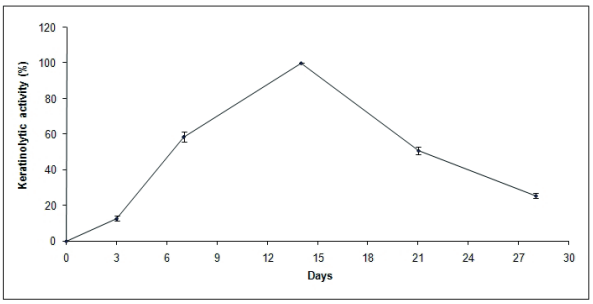

Figure 2. Keratinase production at different incubation periods

Table 1. Purification scheme of keratinase produced by *Cunninghamella echinulata.*

Purification step	Volume (ml)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Fold Purification	Recovery (%)
Supernatant	100	78.2	29.20	2.68	-	100
Acetone	10	44.96	5.18	8.68	3.24	57.4
Affinity chromatography	2	31.07	0.88	35.30	13.17	39.73

On comparison with the standard molecular weight markers, the apparent molecular weight of keratinase was found to be approximately 33 kDa (Figure 3).

Effect of pH and temperature on keratinase activity

The effect of temperature and pH on enzyme activity was determined for partially purified enzyme. The optimum temperature was found to be 30°C and 60°C (Figure 4), and optimum pH was found to be 4.5 and 10.0 (Figure 5) using keratin solution as substrate.

Effect of metal ions and group specific reagents

The effect of various metal ions was tested. It was found that in the presence of Mn^{2+}, activity of keratinase was found to increase three-fold, while Fe^{2+}, Cu^{2+}, Zn^{2+}, Ca^{2+}, Mg^{2+}, Ba^{2+}, Ag^{2+}, and K^{+} were found to increase keratinase activity to different levels. Hg^{+} and Na^{+} inhibited keratinase, and Hg^{+} was found to be a more potent inhibitor compared to Na^{+} (Figure 6).

Among the various group specific reagents PMSF completely inhibited keratinase activity followed by 42% residual activity in the presence of NEM, TLCK, IAA, NAI, SDS, NaI and urea were also found to be inhibitors of enzyme activity. It was highly stimulated by the presence of reducing agents like β-mercaptoethanol and DTT with three-fold enhancement in the presence of β-mercaptoethanol. EDTA displayed no effect on activity (Figure 7)

Discussion

There has been a lot research going on regarding identification, characterization and production of keratinases from keratinophyllic microorganisms. Three keratinase producing fungal species were isolated from three different poultry dump yards, around Bangalore. The microorganisms so isolated were grown on medium containing feather as sole carbon source. Cao showed the isolation frequency of different fungi as follows: *Trichoderma sp.* (100%), *Aspergillus sp.* (77%), *Fusarium sp.* (48%), *Penicillium sp.* (27%), non-sporulating fungus (17%), and bacteria (10%). As described by Onifade *et al.* [22] and Farag and Hassan [12], most of the keratinolytic fungi are belonging to fungi imperfecti. The identification and characterization of new fungal species able to degrade keratinous waste may help to understand its role in nature.

Maximum enzyme activity was observed on day 14 for *C.echinulata*. The kinetics of enzyme synthesis in *Aspergillus flavus* [23] demonstrated that production of the enzyme reached a maximum at day 16, and thereafter

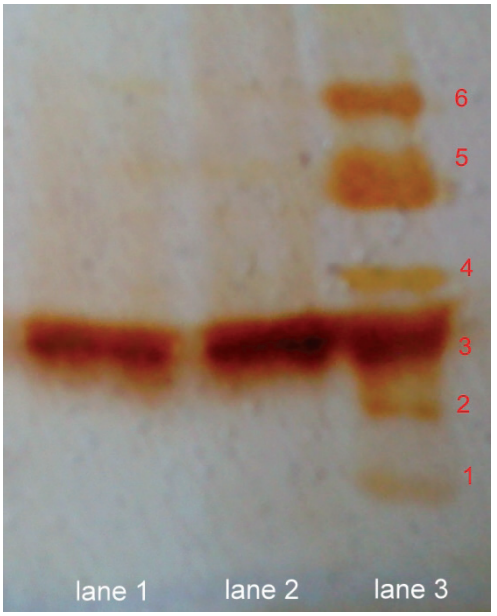

Figure 3. SDS-PAGE analysis during enzyme purifi cation [Lane 1- Partially Purified Sample, Lane 2- Purified Sample, Lane 3- Molecular Weight Markers; 1-5 : Molecular weight markers from 14.4kDa to 97.4kDa].

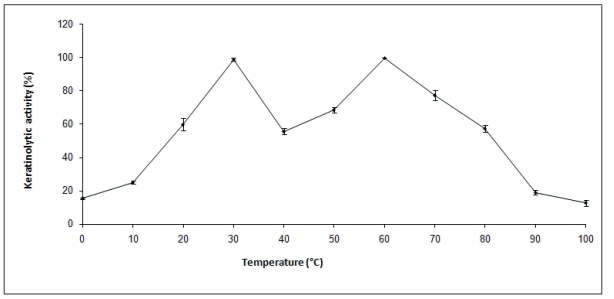

Figure 4. Effect of different temperatures on the activity keratinase from *C.echinulata*

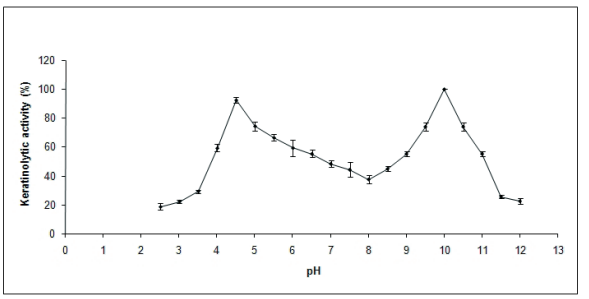

Figure 5. Effect of different pH on the enzyme activity

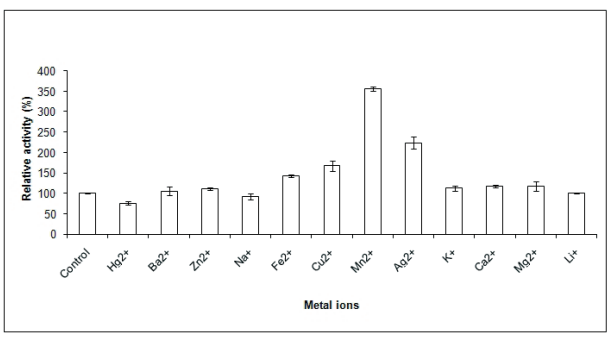

Figure 6. Effect of various metal ions on keratinase activity

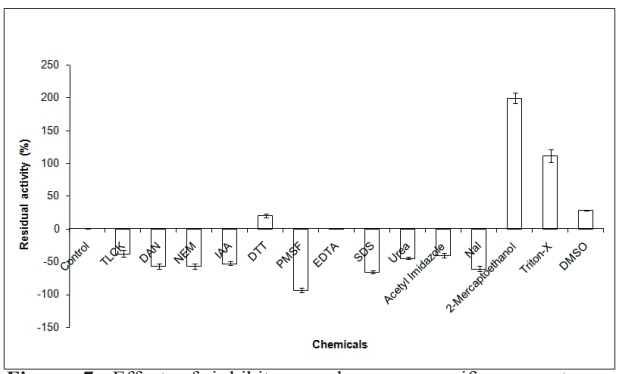

Figure 7. Effect of inhibitors and group specific reagents on keratinase activity

remained stable until day 21. In addition, the biomass increased steadily with incubation time. Similar production kinetics of keratinase has been reported for fungi such as *Endothia parastica* [24], *Trichophyton simi* [25] and *Malbranchea gypsea* [26]. In *A.fumigatus* [27] and *A.oryzae* [28], the rate of keratinase production reached a maximum concentration after 21 days of incubation.

It was seen that the specific activity and fold purification of the enzyme increased with each purification step proving that acetone precipitation and affinity chromatography are suitable easy methods of keratinase purification. A 13.2 fold purification and recovery of 39.7% was seen for lectin agarose column chromatography. Similar results were observed by Thanaa *et al.* [20] in *A.oryzae* with fractional precipitation of 60-80% chilled acetone (-20°C) and it showed a 2.3 fold purification and recovery of 37.9% for DEAE ion exchange chromatography.

Acetone precipitated sample, however, showed one clear band and a number of diffuse bands of different proteins. On comparison with the standard molecular weight markers, the apparent molecular weight of keratinase was found to be approximately 33 kDa. Similar results were obtained for keratinase of *A. flavus* [23] which was estimated to be 31 kDa by both SDS-PAGE gel electrophoresis and zymograms. The molecular weight is in the range of other reported keratinases, between 16kDa [29] and 440kDa [30]. Most of the keratinases also have a molecular weight around 30kDa [31, 29]. Gradisar *et*

al. [32] purified keratinases for non-pathogenic fungi: 33kDa for *Paecilomyces marquandii,* 30kDa for *Doratomyces microsporus* and 22kDa for *A.flavus* keratinase.

The optimum temperature was found to be 30°C and 60°C for *C.echinulata* keratinase. Thanaa *et al.* [20] have shown that 70°C was the optimum temperature of enzyme activity. The high keratinase activity at the temperature is in agreement with some other investigations, which found the optimum temperature to be 50°C. Most keratinases possess an activity optimum in the range of 30-80 °C; for example, keratinase from *B. Pseudofirmus* AL-89 is of 60~70 °C [33], *B.licheniformis* [34] and *Nocardiopsis* sp. TOA-1 [35] is of 60°C and a few have an exceptionally high temperature optimum of 100°C [10].

C. echinulata keratinase has an optimum pH of 4.5 and pH 10.0 signifying that the enzyme can be used at both acidic and basic pH. This could be because *C.echinulata* produces two isoforms of keratinase. Similar results were obtained by Korkmaz *et al*. [34] which indicated that the *Bacillus licheniformis* enzyme was active over a wide range of pH with optimum 11.0 and temperature with optimum 60°C.

Our results also showed that the presence of Mn^{2+} increased the activity of keratinase three-fold. Hg^{2+} and Na^{+} inhibited keratinase, and Hg^{2+} was found to be a more potent inhibitor compared to Na^{+}. Similar results were obtained by Tapia and Simoes [36], who also found that the enzyme was inhibited by Hg^{2}+. Keratinase inhibition by Hg^{2+} may suggest that a free cysteine is present at or near the active site. It has been suggested that inhibition by Hg^{2+} is not just related to binding of the thiol groups but may be a result of an interaction with tryptophan residues or with the carbonyl group of amino acids in the enzyme [37, 38].

C. echinulata keratinase was inhibited by PMSF, thus it may be a serine protease. Enzyme activity was inhibited by group specific reagents like TLCK, IAA, NEM and NAI indicating that serine, cysteine, tyrosine and lysine could be present in the catalytic site. EDTA displayed no effect on activity of the enzyme, thus it does not have any divalent ion requirement. In contrast, Cai *et al.* [17] found that EDTA had a positive effect on the keratinase activity. Also, the enzyme produced by the strain *A.oryzae* seems to belong to the metalloprotease type since it was inhibited by EDTA [20].

Keratinase from *C. echinulata* was highly thiol activated as its activity was enhanced in the presence of DTT and β-mercaptoethanol. Thiol activation of keratinases has been reported earlier, and it is a positive attribute for their action on cysteine rich keratin substrates [2, 20].

Keratin hydrolysis by microorganisms is reported to not simply rely on the production of keratinolytic proteases. Release of thiol groups during microbial growth on keratinous materials supports the essential role of the reduction of disulfide bonds for efficient keratin degradation. Production of intra- and/or extra-cellular disulfide reductases, release of sulfite and thiosulfate, and also a cell-bound redox system are reported to lead to sulfitolysis [39].

Overall, the present keratinase is an alkaline, thiol- and metal-activated, and thus can find applications in various industries especially in detergent and leather industries. It has considerable biotechnological potential for the processing of poultry feather waste and in microbiological keratin hydrolysates for feedstuffs, fertilizers, glues and foils or is used for the production of amino acids and peptides.

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