



Screening, Partial Purification and Characterization of Keratinase from Newly Isolated Marine Fungi

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ABSTRACT

A feather degrading fungi was newly isolated from marine sponges collected from Kulasekarapattinam, Tuticori district, Tamil Nadu using agar mineral medium containing soluble keratin as a substrate and it was identified by its morphological features as *Scopulariopsis brevicaulis*. The selected growth medium contained feather meal as a sole source of carbon and nitrogen at pH 7.5 and temperature at 28±2°C. Purification was carried through ammonium sulfate precipitation and chromatography via Sephadex G-100 column using with soluble keratin as a substrate, 10.0-fold purification with a yield of 22.8% was obtained. The partially purified keratinase has a molecular mass of 28±0.5 kDa, optimum pH in the neutral region 7 to 7.5 and optimum temperature of 50°C. The keratinases produced by the fungi could be useful in decomposition of keratin-wastes and could find applications in leather, pharmaceutical and cosmetics industries as well.

Keywords: Decomposition, Keratin, Keratinase, Marine sponges, Purification, *Scopulariopsis brevicaulis*.

INTRODUCTION

Keratin is a highly stable, insoluble fibrous protein composed of tightly packed α -helix (α -keratin, e.g. hairs) or β -sheet (β -keratin, e.g. feathers) intertwined polypeptide chains which form dense network of intermediate filaments (IF).¹ There are approximately 30 different keratins which are generally grouped into epithelia keratins (in epithelia cells) and trichocytic keratins which make up hair, nails, horns and reptilian scale. Keratins are also classified as hard (those containing up to 5 % sulphur) and soft (those containing up to 1 % sulphur) keratins.² Different approaches to dispose feather waste, including land filling and burning but these techniques can cause contamination of air, soil and water.³

Feathers can be hydrolyzed by mechanical or chemical treatment to feedstuffs, fertilizers, glues and foils for the production of amino acids and peptides but these techniques lead to environmental pollution. There are several traditional ways to degrade feathers such as alkali hydrolysis and steam pressure cooking but these techniques may not only destroy the amino acids but also consume large amounts of energy.⁴ Degradation of poultry feathers by keratinolytic proteases offers an alternative method for efficient bioconversion, nutritional enhancement and environmental friendliness.⁵

Keratinases (E.C.3.4.21/24/99) are a particular class of proteolytic enzymes that display the capability of degrading insoluble keratin substrates.⁶ These enzymes are gaining importance in the last years, as several potential applications have been associated with the hydrolysis of keratinous substrates among other

applications. A significant amount of fibrous insoluble protein in the form of feathers, hair, nails, horn, and other are available as byproducts of agro industrial processing.⁷ These keratin-rich wastes are difficult to degrade as the polypeptide is densely packed and strongly stabilized by several hydrogen bonds and hydrophobic interactions, in addition to several disulfide bonds.

Keratinases are used in leather industry, in textile industry, as a feed hydrolysate, as a fertilizer, as a detergent aids to remove stains, for production of biohydrogen, in ungual drug delivery, to hydrolyse prion protein.⁸ Because of these potential applications of keratinases, this study was undertaken to screen fungi from marine source that produces a highly active keratinase, to partially purify and characterize the enzyme.

MATERIALS AND METHODS

Isolation of fungi from marine samples

The marine sponge samples were collected from south-east coast of INDIA, in 100 ml sterilized screw cap bottles contains ISP2 (YEME broth) medium. A quantity of 1 ml sponge extract was aseptically transferred to 50 ml (stock) of sterilized seawater in a 250 ml Erlenmeyer flask and allowed for aeration and agitation on orbital rotary shaker at 200 rpm for 30 minutes at room temperature. A series of culture tubes containing 9 ml of sterile seawater was taken. From the stock culture, 1 ml suspension was transferred aseptically to the 1st tube (10^{-1}) and mixed well. Further serial dilutions were made to produce up to 10^{-10} suspensions. Plates containing yeast extract agar medium supplemented with the antibiotic rifampicin and

was added to each agar plate to minimize the growth of gram negative and gram positive bacteria and yeast contaminants respectively. The inoculated agar plates were incubated at 28°C ($\pm 2^\circ\text{C}$) for 7 to 14 days and examined the plates for the presence of developing colonies of fungal hyphae on daily. Fungi were recognized by their characteristic branched vegetative mycelia, and, when present, aerial mycelia and spore formation. The obtained fungal isolates were maintained as pure culture and used for further study.

Screening on agar plates for keratinase activity and identification of isolate

The ability to degrade the keratin on solid medium was done following method of Wawrzkiwicz.⁹ Chicken feathers of white colour were dissolved in dimethyl sulphoxide (DMSO) and re-precipitated with acetone. The precipitated keratin was added to the sterile agar medium. Keratinase activity was detected as formation of clear zone around the colony following incubation for 6 day at room temperature and the diameter of the clear zone was recorded. The colony with highest keratin hydrolyzing ability was picked up and purified by repeated screening on the same medium. Pure culture was maintained on Sabouraud dextrose agar slants at 4°C. The fungal culture was identified by Microbial Type Culture Collection, Chandigarh, India.

Preparation of feather meal

Feather meal was prepared by slightly modified method of Minghai Han.¹⁰ The fresh feathers from local market were washed with tap water, cut into sections of approximately 5 mm and sterilized by autoclaving at 121°C for 2h, then further dried at 60°C for 24 h. The dried feathers were milled into powder.

Production of keratinase enzyme

The primary inoculum of *S.brevicaulis* was prepared by incubating a loop full of fungal spores in the Sabouraud's dextrose agar containing (g/l) Peptone-10.0; Dextrose-40; Yeast extract-5; Agar-20; Distilled water-500ml; Sea water-500ml; pH-6.6 for about 5 days. For enzyme production, 5% of the inoculum was transferred to the production medium which was the 50ml of minimal medium containing (g/l) MgSO₄.H₂O-0.5; KH₂PO₄-0.1; FeSO₄.7H₂O-0.01; ZnSO₄.7H₂O-0.005; Feather meal-1%; Distilled water-500ml and Sea water-500ml; P^H adjusted to 7.5 and the flasks were incubated up to 7 days at room temperature (28°C \pm 2°C) on a rotary shaker with constant shaking at 120 rpm.¹¹ The culture filtrates from 5th day were collected from each set of flasks by separating the mycelia by centrifugation and stored at 4°C. The culture filtrates were used as the enzyme source.

Keratinolytic activity determination

The keratinolytic activity was assayed as follows: 1.0 mL of crude enzyme properly diluted in Tris-acetate buffer (0.2 M, pH 7.0) was incubated with 1 mL keratin solution at 50°C in a water bath for 30 min, and the reaction

mixture was stopped by adding 2.0 mL trichloroacetic acid (15%). After cooling, centrifugation at 5000 \times g for 10 min, the absorbance of the supernatant was determined at 280 nm against a control. The control was prepared by incubating the enzyme solution with 2.0 ml TCA without the addition of keratin solution. One unit (U/ml) of keratinolytic activity was defined as an increase of corrected absorbance of 280 nm (A₂₈₀) (modified method by Gradisar)¹² with the control for 0.01 per minute under the conditions described above and calculated by the following equation:

$$U = 4 \times n \times A_{280} / (0.01 \times T)$$

Where n is the dilution rate; 4 is the final reaction volume (ml); T is the incubation time (min).

Protein determination

The protein content of the enzyme was determined by Folin-Phenol reagent¹³, using bovine serum albumin as a standard.

Preparation of keratin solution

Keratinolytic activity was measured with soluble keratin (0.5%, w/v) as substrate. Soluble keratin was prepared from white chicken feathers.¹⁴ Native chicken feathers (10g) in 500 mL of dimethyl sulfoxide were heated in a reflux condenser at 100°C for 2 h. Soluble keratin was then precipitated by addition of cold acetone at -20°C for 2 h, followed by cooling centrifugation at 8050 \times g for 10 min. The resulting precipitate was washed twice with distilled water and dried at 40°C in a vacuum dryer.

Purification of keratinase

The crude enzyme was precipitated by salting out using ammonium sulfate. The calculated amount of ammonium sulfate was added gradually to the supernatant with stirring, to obtain 80% saturation, incubated for 12 h at 4°C. After centrifugation at 6000 g for 15 min, the precipitated proteins were collected in a minimum volume of 0.2M Tris-acetate buffer (pH 7.0), and then dialyzed against the same buffer for 12 h. The dialysis step was repeated (three times) for the enzyme preparation under the same conditions, until there was total removal of ammonium sulfate as checked by barium chloride. The preparation was concentrated by lyophilization.

Next purification step of the concentrated sample was carried out through a sephadex G-100 gel filtration column equilibrated with 20 mM Tris-HCl buffer (pH 9.0). The keratinolytic active fractions were eluted with the same buffer, pooled using keratin-agarose plate assay and concentrated. All purification steps were performed at room temperature.

Keratin-agarose plate assay

A method that was an alternative to the keratin hydrolysis assay was used to screen large numbers of fractions collected from Sephadex G-100 column. This was modified method of Xiang Lin¹⁵ who was used for

screening of fractions for proteolytic activity. Two grams of agarose was dissolved in 98ml of 20 mM Tris-HCl buffer (pH 9.0). After the agarose solution was cooled to 60 to 70°C, 500 mg of soluble keratin powder was added and mixed with gentle agitation. The mixture was poured rapidly onto a clean glass plate (12 inches) and spread evenly. After the agarose had solidified, small wells (5 mm in diameter) were punched in the agarose. A 20µl of a sample from each fraction was applied to each well. The plate was incubated in a moist atmosphere either overnight at 37°C or for 4 h at 50°C. Clear zones around wells were indicative of keratinolytic activity. Same diameter containing zones were pooled and concentrated. A similar method was previously published by Sandholm.¹⁶

Molecular weight determination

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

In order to determine molecular weight, the partially purified enzyme and known molecular weight markers were subjected to electrophoresis. SDS-PAGE was performed with 12% polyacrylamide gels¹⁷ and the protein bands were stained with a solution of Coomassie blue R-250.

Effects of pH and temperature on enzyme activity

Effects of pH and temperature on the keratinolytic activity were determined using keratin as substrate. Keratinolytic activity was studied in the pH range of 3 to 9 using 20mM Tris-acetate buffer. 1 ml of keratin solution was then added to each test tube and incubated for 30 min at 50°C. The assay was performed by pre-incubating 1mg/ml of enzyme in each of the pH buffer. The reaction was arrested by adding 15% TCA and the activity was determined as described earlier. The optimum temperature for keratinolytic activity was determined by performing the enzyme reaction at incubation temperatures between 30°C and 80°C.

RESULTS AND DISCUSSION

Isolation of fungi from marine sponges

A total of 21 marine fungi were isolated (Figure 1) from 8 different marine sponges, collected from Kulasekarapattinam, Tuticori district, Tamil Nadu in sterile screw capped bottles containing YEME medium.

Among 21 isolates four isolates were showing keratinolytic activity. From those one isolate was selected which was having significant keratinolytic activity, and it was used for further study. The colonies that showed a clear zone around them after addition of TCA solution on keratin agar plates (Fig. 2) were regarded as enzyme producers. Based on its morphology (Fig. 3) it was identified as *Scopulariopsis brevicaulis*, with ref. MTCC11794 by Microbial Type Culture Collection, Chandigarh, India. Keratinase production from *Scopulariopsis brevicaulis* was studied by Anbu¹⁸ Eman and Neveen¹⁹ and Malviya²⁰. They isolated from soil and

from poultry farm. But in this present study we isolated the fungi from marine sponges which requires both sea water and distilled water for its growth and enzyme production.

Initial screening of fungal isolates



Figure 1: Crowded Plate of fungi

Screening on agar plates for keratinase activity and identification of isolate

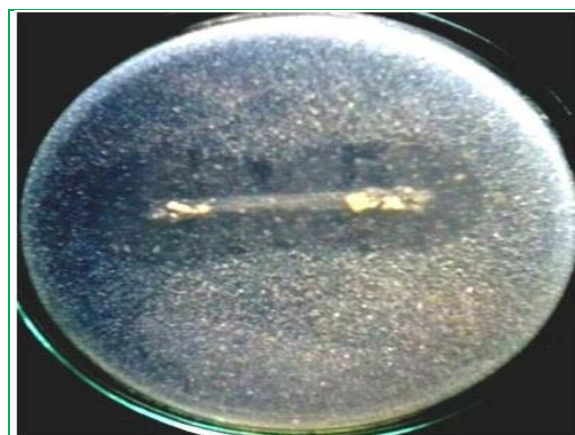


Figure 2: Keratinolysis by selected fungi

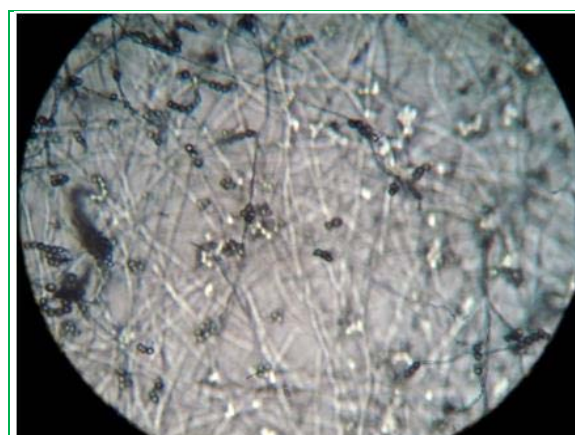


Figure 3: Microscopy of fungi

Production of keratinase

Insoluble feather meal 1% concentration was used as substrate which can act as a whole carbon and nitrogen source and incubated with the keratinase producing

Scopulariopsis brevicaulis. The supernatant demonstrated an increase in the absorbance at 280 nm against control.

Purification of keratinase

A summary of the purification of keratinase from the culture medium of *Scopulariopsis brevicaulis* is presented in Table 1. Centrifugation, salt precipitation, dialysis, and sephadex G-100 gel chromatography yielded a purified keratinase fraction having an overall purification factor of

10-fold. The final product had a specific activity of about 80 U/mg and yield of 22.44%. Jeong²¹ purified keratinase from *Aspergillus flavus* strain K-03 with 11 fold purification. Yassin²² purified keratinase from *Aspergillus flavipes* with an overall purification factor 2.67-fold. Elution from the Sephadex G-100 column yielded a homogeneous protein, as shown by a single protein band in SDS-PAGE. Purified fractions were pooled by keratin-agarose plate assay showed in Figure 4A.

Table 1: A summary of the purification of keratinase from the culture medium of *Scopulariopsis brevicaulis*

Purification steps	Total activity (IU)	Total protein (mg)	Specific activity (IU/mg)	Purification fold	Yield (%)
Fermented broth	60.6	7.21	8.4	1	100
Ammonium sulphate Precipitation	25.3	1.63	15.52	1.85	41.75
Dialysis	23.6	0.65	36.3	4.32	38.94
Sephadex G-100	13.8	0.17	81.17	9.66	22.8



Figure 4A: Highest zone diameter produced (16, 17, 18, 19) fractions were pooled, concentrated and used for SDS-PAGE analysis

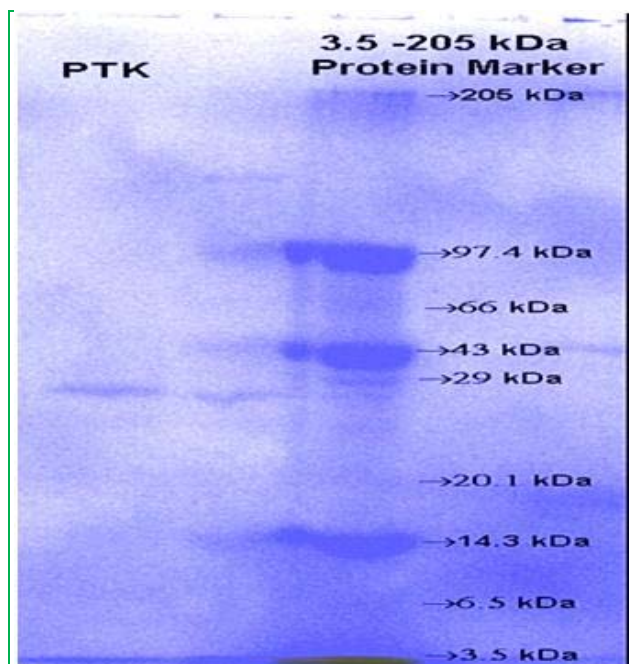


Figure 4B: SDS-PAGE of keratinase

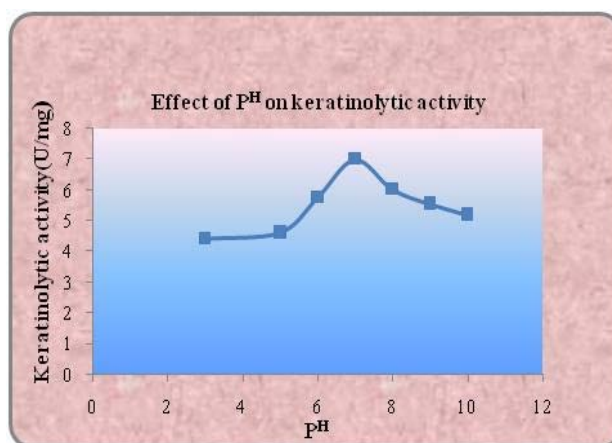


Figure 5: Effect of P^H on keratinase activity

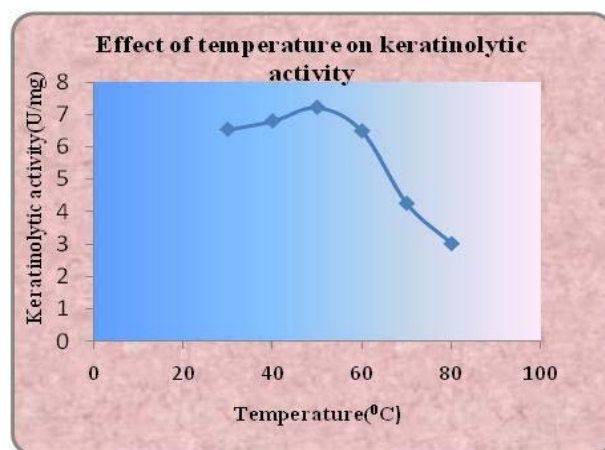


Figure 6: Effect of temperature on keratinase activity

Determination of molecular weight

The molecular weight of the purified enzyme was estimated to be about 28 ±0.5 kDa (Figure 4B). Different molecular weights of extracellular keratinases could be detected for different isolates of the same fungal species. For example, the purified keratinase of a *S. brevicaulis* isolate was homogenous showing a single protein band in SDS-PAGE with a molecular weight of 39 kDa.²³ On the

other hand, SDS–PAGE of two purified extracellular keratinases (KI and KII) from another isolate of the same fungus produced only one band each, suggesting homogeneity. Estimated molecular weights were 45–50 kDa and 24–29 kDa for KI and KII, respectively.²⁰ The purified enzyme is similar to the molecular weight of the enzymes from *S.brevicaulis*-28.5kDa¹⁹, *Trichophyton mentagrophytes* – 28–65 kDa²⁴ and *M. canis* – 31.5–43.5 kDa.^{25,26}

Effect of pH and temperature on partially purified enzyme activity

The maximum activity of the keratinase from *S.brevicaulis* was observed at pH 7.0 (figure 5) and 50°C (figure 6) using keratin solution as substrate and retains its stability up to PH- 9.0. Anbu²³ reported maximum keratinase activity at pH-8.0 and 40°C with *S.brevicaulis* isolated from poultry farm soil. P^H 7.8, optimum temperature 40°C and 35°C were reported by Malviya.²⁰ For K-I, K-II purified from *S. brevicaulis*. The purified keratinase is thus a better thermotolerant and alkaliphilic enzyme.

CONCLUSION

Keratinase production by the feather degrading fungus *S.brevicaulis* which was isolated first time from marine sponges was investigated and the keratinolytic activities were further characterized. Efficient feather hydrolysis by the fungus revealed production of good amount of keratinase activity with broad optimum pH and higher temperature optima which could find application in converting the large amount of waste generated from poultry into digestible animal feed. Since, this fungus uses feathers as the sole sources of carbon and nitrogen, the production of enzyme will be cost effective.

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