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PURIFICATION AND CHARACTERIZATION OF KERATINASE FROM NATIVE FEATHER-DEGRADING *Streptomyces albus*

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ABSTRACT

The keratin occurs naturally in the form of feathers, hair, nails and wool all over the world. As the physiological and chemical methods of keratin degradation are not easy possible, the biological method has gained importance. It can be biodegraded by some Keratinolytic *Streptomyces* sp. The present study investigated purified keratinase from Keratinolytic *Streptomyces albus*. The cell-bound keratinolytic enzyme was purified 28.91 fold by gel filtration chromatography. The enzyme was characterized as a serine protease with a molecular mass of 40-45kD. Optimal activity pH and Temperature were measured at 7.0 and 40^0C, furthermore the various inhibitors had different effect on enzyme activity. PMSF and heavy metal ion Hg^{+2} were the most potent inhibitors and EDTA induced the activity by more than 142%, 2-mercaptoethanol did not show any impact on the enzyme, where pCMB, KCN, 8-hydroxyquinoline and cystine inhibited activity moderately.

INTRODUCTION

Keratin is an insoluble, high stable protein found mostly in feathers, wool, nails and hairs of vertebrates (Shih, 1993) and then it arises as a waste product in many ways. Feathers are produced in large quantity as a waste by poultry product processing plants; it reaches millions of tons per year worldwide (Fernandes, 2010). A number of keratinolytic microorganisms have been reported, including some species of fungi such as *Microsporum* (Williams *et al.*, 1991), *Trichophyton (*Essien *et al.*, 2009), *Bacillus* (Anbu *et al.*, 2008; Cai and Zheng, 2009; Macedo *et al.*, 2005), *Streptomyces* (Pillai 2008; Syed, 2009; Szabo, 2000) and Actinomycetes (Tatineni, 2008; Bockle, 1995). Keratinases are a group of serine metalloproteases, release the free amino acids from keratinous proteins. Keratin is resistant to the common proteolytic enzymes, papain, pepsin and trypsin (Papadopoulos *et al.*, 1986). These enzymes have been studied for dehairing processes in the leather industry (Raju *et al.*, 1996) and hydrolysis of feather keratin (Lin *et al.*, 1995), which is a by-product generated in huge amounts by the poultry industry. Discarded feathers are currently used to produce feather meal through thermal processing, resulting in a low nutritional value product (Wang and Parsons, 1997). Feather hydrolysates produced by bacterial keratinases have been used as additives for animal feed (Williams *et al.*, 1991). Keratinolytic enzymes have found important utilities in biotechnological processes involving keratin-containing wastes from poultry and leather industries, through the development of non-polluting processes. After hydrolysis, the feathers can be converted to feedstuffs, fertilizers, glues, films and as the source of rare amino acids, such as serine, cysteine and proline (Gupta and Ramnani, 2006; Cai *et al.*, 2009). Considering their high content of useful compounds, animal wastes have a great potential for many applications. The present paper reports on the optimization of methodology for keratinase production, purification and its characterization using locally isolated *Streptomyces albus* a thermotolerant actinomycete.

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MATERIALS AND METHODS

Microorganism and inoculum preparation

A novel thermophilic actinomycete was isolated from the hot soil zone of Gulbarga and Bellary, identifies as Streptomyces albus and used for keratinase production. The spore suspension was prepared by scrapping the spores of *Streptomyces albus* from 7 days old culture grown on aspergine agar medium (Sreenivasa *et al.*, 2003). The spore concentration was adjusted to 5X106/ml.

Determination of keratinase activity

The keratinase activity was assayed by the modified method of Cheng *et al*. (1995) by using keratin as a substrate with slight modification. The reaction mixture was prepared by mixing 1ml of 1% keratin in phosphate buffer (pH 8.0) and 0.5 ml of enzyme solution and incubated at 30^0C for 30 min. After incubation, the reaction was terminated by adding 2 ml of 10% trichloroacetic acid (TCA). After the separation of untreated keratin precipitate by centrifugation, 1ml of clear supernatant was mixed with 5ml of 0.4 M Na_2CO_3 and 0.5 ml of Folin-Ciocalteau's phenol reagent. After 30 min, absorbance was measured at 660 nm against blank. All assays were done in triplicate. One unit of keratinase activity was defined as the amount of enzyme that released one micromoles of tyrosine per minute under the standard assay conditions.

Determination of Protein content

The protein content of the enzyme extract was determined by Folin-Phenol reagent (Lowry et al., 1951), using bovine serum albumin as a standard.

Purification and Characterization of Keratinase

The *Streptomyces albus* were grown in optimized starch casein agar media and incubated in static condition at room temperature. The culture fluid of feather was harvested up to 20th day of incubation, the point of view maximal enzyme activity.

Ammonium sulphate precipitation and dialysis

The crude extract fluids (200 ml) were concentrated by 80% saturation using ammonium sulphate. The protein precipitate obtained was separated by centrifugation at 10,000 rpm for 10 min and the pellet was dissolved with minimum volume of phosphate buffer (56 mM, pH 7.8). The dissolved sample was dialyzed (Cellophane membrane, Sigma) against 5 mM phosphate buffer (pH 7.8) for 8 h.

Gel filtration

After dialysis, the sample (3.0 ml) was subjected to gel filtration fractionation with a sephadex G-100 column that had been equilibrated with 0.056 M phosphate buffer (pH 7.8). Elution was conducted at a flow rate of 15ml/h and 3ml of fractionation collected. The major peaks of keratinase were detected and the fractions containing these peaks were pooled separately. These pools were lyophilized for further purification.

Polyacrylamide gel electrophoresis (PAGE)

Preparative polyacrylamide gel electrophoresis with 7.5% gel was conducted to detect keratinase in pooled purified fractions. Protein bands were visualized with silver staining.

Effect of pH and temperature on keratnase activity

Keratinolytic activity of purified enzyme was measured in the range of pH 4.0 to 11.0 using fallowing buffers: sodium acetate (pH 4.0-6.0), sodium phosphate (pH 7.0-8.0), and Tris-NaoH buffer (pH 9.0-11.0). The optimum temperature was determined by incubating reaction mixture at different temperature range from 20^0C to 80^0C for 20 min.

Effect of inhibitors

Purified *Streptomyces albus* keratinase was pre-incubated with each inhibitor at pH 7.0 for 1 h at 40^0C and then assayed for residual activity. The inhibitors used were: EDTA, pCMB, KCN, $HgCl_2$, cystine, PMSF, 2-mercaptoethanol and 8-hydroxyquinoline.

RESULT AND DISCUSSION

Purification of Keratinase

The strain of *Streptomyces albus* grew well and completely degraded chicken feathers in the culture broth (Fig 1). This intense feather-degrading activity was achieved in the range of 25-37ºC and with initial pH adjusted from 6.0 to 8.0. Keratinase was extracted from culture broth and further purified.

A **B**

Fig. 1. Complete feathers degradation by *Streptomyces albus*

(A) Feathers control without *Streptomyces albus*,
(B) The feathers with *Streptomyces albus*.

The keratinase from feather degraded media was subjected for ammonium sulphate precipitation, dialysis and gelfiltration chromatographic purification. In the feather sample, the crude enzyme exhibited 8.7×10^{-3} U/mg of specific activity (Table-1). In the ammonium suplhate saturated enzyme 14.5×10^{-3} U/mg activity was found and the specific activity of enzyme after dialysis was 21.5×10^{-3} U/mg. The gelfiltrtion purification led to 25.16 U/mg keratinase. Over all purification fold achieved was 28.91. Similar purification protocols reported used for keratinase from the solid cultures of bacterial species such as *Fervidobacteium* (Nam *et al*., 2002), *Chrysobacterium* sp. Kr6 (Riffel *et al*., 2007) and *Streptomyces* sp. (Bockle *et al*., 1995).

Table 1: Purification of extra cellular keratinase from *S. albus*

Sl. No	Fractionation step	Total protein (mg)	Total activity (kU)	Specific activity X 10^{-3} (U/mg)	Recovery (%)	Fold Purity
1	Crude culture fluid	71.0	0.62	8.7	100.0	1
2	Ammonium sulphate precipitation	37.0	0.54	14.5	86.7	1.66
3	Dialysis	19.0	0.41	21.5	76.4	2.47
4	Sephadex G-100 fractionation	3.1	0.78	25.16	71.2	28.91

The elution pattern of gel filtration and purification of keratinase from feather samples clearly demonstrated that, the enzyme was eluted in early fractions (Fig 2).

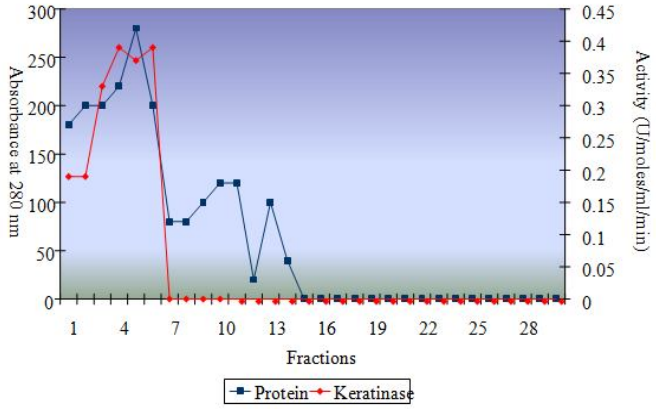

Fig. 2: Gel filtration elution profile of keratinase

The sharp peak of activity was found along with the highest protein eluted samples. It was interesting to determine the elution of the enzyme in single peak in feather samples. The SDS-PAGE analysis of purified keratin from feather showed protein bands of 40-45 kD respectively (Fig 3). Keratinase activity (2.45kU/m) in crude culture extracts was observed by *Streptomyces albidoflavus* (Bressollier *et a*l., 1999), *Kytococcus sedentarius* (Longshaw *et al*., 2002) and *Trichophyton rub-rum* (Sanyal *et al.,* 1985). Concentration of the culture fluid by vacuum evaporation resulted decrease in keratinase activity. A similar result was observed following dialysis of enzyme samples with phosphate buffer (pH 7.8), even though the specific activity was increased. The elution pattern of the keratinase isolated from feather displayed a single peak of activity (Malviya *et al.,* 1992). The PAGE analysis of pooled eluents from gel filtration chromatography showed the presence of single band; the keratinase purified (Yassin *et al*., 2012 and Pushpalata 2010) had two subunits of 50 and 29 kD.

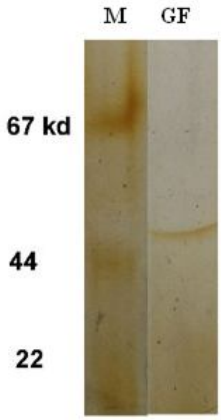

Fig. 3: SDS- PAGE of purified keratinase from *S. albus*

M: (Protein) Molecular weight marker (medium range, Bangalore Genie, Bangalore); GF- gel filtration purified kertinase

The optimum pH for the activity of keratinase isolated was of 7.0 (Fig 4). This result is in agreement with those described for most feather-degrading *Bacillus* (Santos et al., 1996; Brandelli and Riffel, 2005). For production of keratinase by *B. licheniformis* and a recombinant *B. subtilis*, uncontrolled pH operation was more favorable than the controlled pH operations (Wang and Shih, 1999). Similar findings were observed for alkaline protease by *B. licheniformis* (Calik *et al*., 2002). The keratinase sample, showed a gradual increase in the activity with increasing pH up to the optimum as followed by a gradual fall in the activity. The keratinase studied by Malviya *et al*., (1992) was also showed an optimum pH at 7.8.

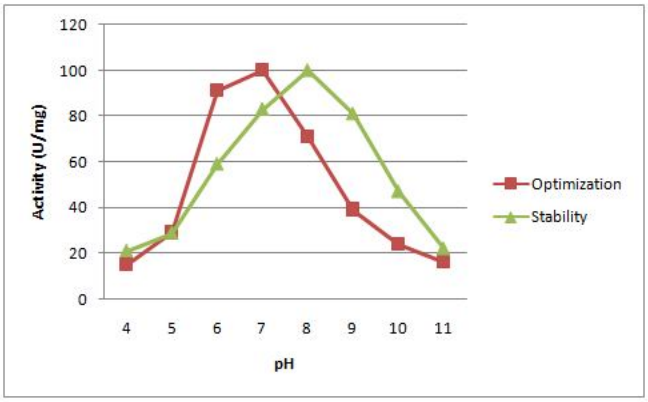

Fig 4: Effect of pH on activity and stability of keratinase from *S. albus*

The enzyme stability studied at various pH ranging from 4.0 to 11.0 showed a significant increase in the stability up to pH 8.0 (Fig 4), which declined thereafter. 100 percent stability was recorded at pH 8.0. The keratinase subunits analyzed by Malviya *et al.,* (1992) were stable between pH 5.0-7.8. However, the keratinase of *S. brevicaulis* were most active at alkaline pH. The phenomenon was similar to dermatophytic keratinase that degrade human hair keratin (Sanyal *et al.,* 1985), wool (Weary *et al.,* 1967), bovine hoof and horn (Meevootisom and Niederpruem, 1979). The effect of temperature on keratinase was studied from 20^0C to 80^0C. The activity increased from 20^0C up to 80^0C, but optimum temperature for the activity recorded at 40^0C respectively (Fig 5). Proteases from *Chryseobacterium* sp. are often produced at mesophilic temperatures. A metalloprotease of *C. indologenes* Ix9a (Venter *et al.,* 1999) and an endopeptidase of *Chryseobacterium* sp. (Lijnen *et al.,* 2000) were produced during cultivation in nutrient broth at 25ºC and 28ºC. Although those conditions were considered satisfactory to produce proteolytic activity and other settings were not investigated. Although keratinolytic bacteria often display optimal growth and activity at higher temperatures (Lin *et al.,* 1999; Kim *et al.,* 2001), this is consistent with optimum values

described for keratinolytic gram-negatives such as, *Vibrio* sp. kr2 (Sangali and Brandelli, 2000), The karatinase from *Chryseobacterium* sp. had optimum temperature of 30^0C (Brandelli and Riffel, 2005). *Lysobacter* sp. (Allpress *et al.*, 2002) and *Stenotrophomonas* sp. D-1 (Yamamura *et al.*, 2002), which showed optimum temperature for growth and keratinolytic enzyme production ranging from 20ºC to 30ºC. The optimum temperature for keratinase activity reported by Malviya *et al.*, (1992) was 40^0C and 35^0C, though K-I was more stable over a broader temperature range than K-II. The temperature stability decreased rapidly as temperatures increased above 40^0C, similar to our observations.

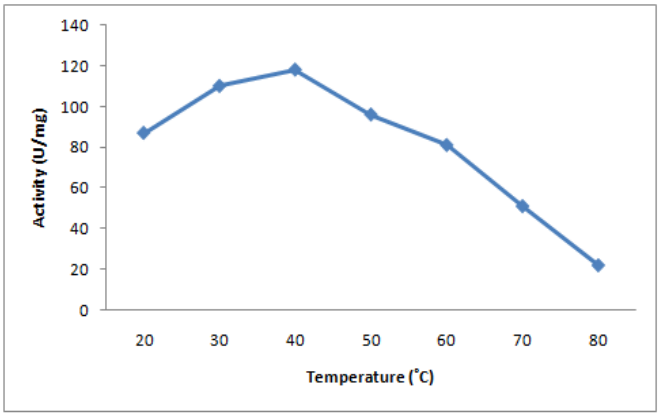

Fig. 5: Effect of temperature optimization on activity of keratinase from *S. albus*

The various inhibitors had different effect on enzyme activity (Table 2). The PMSF was found most potent inhibitor, as it completely killed the activity of enzyme, indicating serine residues at the active site of the Keratinase (Rajak *et al.*, 1992). Similar observations were made by Bernal, et al, (2006) for one of the subunits of keratinase. Several workers have reported the production of serine proteases by keratinophilic fungi (Sanyal *et al.*, 1985; Grzywnowicz *et al.*, 1989; Rajak *et al.*, 1992). It signifies that the keratinase is a protease, as PMSF is known to act only on proteases. The $HgCl_2$ as heavy metal ion also inhibited the activity by 100%. The induced activity of enzyme by EDTA, by more than 140% stating that keratinase is a metallo-enzyme. Keratinase from a *Trichophyton* species has shown metal dependence for optimal keratinase activity. But reports of Malviya *et al.*, (1992), Tikiuchi *et al.*, (1982) and Muller and Saenger, (1993), similar result found for a weak inhibition of keratinase by EDTA indicate keratinase is non-metal dependent enzyme. 2-mercaptoethanol did not show any impact on the enzyme, where8-hydroxyquinoline, KCN, pCMB and cystine moderately inhibited the activity.

Close scrutiny of the properties of keratinase revealed that their properties were in many respects identical to the enzymatic activities of dermatophytes (Sanyal *et al.*, 1985; Grzywnowicz *et al.*, 1989). Keratinases have enormous potential applications in processing waste in the poultry and leather industries. The recent finding showed that the keratinase of *B. licheniformis* PWD-1 cause enzymatic breakdown of prion protein Pr PSc (Langeveld *et al.*, 2003) and open a novel relevant application for broad range keratinases. The present study adds to the knowledge of keratinase to take up further study for efficient applications in industries and environmentally.

Table 2: Effects of various inhibitors on the activity from *S. albus*

Inhibitor	Concentration (mM)	Residual activity (%)
pCMB	1	78
PMSF	1	0
EDTA	5	14[illegible]
8-hydroxyquinoline	1	71
2-Mercaptoethanol	2	10[illegible]
$HgCl_2$	1	0
KCN	5	81
Cystine	5	69

Enzyme activity without the addition of inhibitor was considered 100%. All the values are the mean of three independent estimations.

Conclusion

The objective of the present investigation was to optimize conditions for keratinase production and its characterization of native feather degradation. *Streptomyces albus* produce high keratinase activity. It is used for potential applications in processing waste in poultry, leather industries and different fields like for de-hairing and removing of substance like hair, feather and wool (environmental pollutant), the further it is used for investigations of other research areas.

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