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CHARACTERIZATION OF KERATINASE FROM ASPERGILLUS FLAVUS S125

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ABSTRACT

Keratinase enzyme extracted from the isolated fungus Aspergillusflavus S125 was undergone characterization. The properties of the purified enzyme were studied. The enzyme was most active at pH 10. The optimum temperature for maximum keratinase production was 65°C. Among the various salts evaluated for their effect on keratinase activity, none of them enhanced the enzymatic activity. Hg_2^+, Cu_2^+, Fe_2^+ and Zn_2^+ inhibited the activity of the enzyme significantly. The influence of different inhibitors on keratinase activity was investigated using phenyl methyl sulphonyl fluoride (PMSF), iodole acetic acid (IAA), $HgCl_2$, and ethylene diamine tetra acetate (EDTA). Total inhibition of enzyme activity in presence of PMSF was observed. The activity in presence of $HgCl_2$ was negligible. The enzyme maintained more than 80% of its original activity in the pH range 8-11.5. The Km value and V max calculated from the plot were 15.38 mg/mL and 5U respectively. Partial gene sequence of alkaline protease gene from Aspergillusflavus S125 strain (AB 807664) was identified.

Keywords: Aspergillusflavus S125, keratinase, Km value, V max and alkaline protease

Currently, the term 'keratin' covers all intermediate filament-forming proteins with specific physicochemical properties. The protein chains are packed tightly either in α chain (α keratins) or in β sheet (β keratins) structures. Therefore, keratinous material is water insoluble and extremely resistant to degradation by proteolytic enzymes such as trypsin,pepsin, and papain. In human hair keratin is present as α keratin and in bird feathers as β keratin (Kelly et al., 2006).

The accumulation of feather wastes from poultry farms lead to pollution of both air and underground water resources, consequently the disposal of this waste is a global environmental issue (Lin et al., 1992). There is a growing trend to apply cheap and environment friendly methods to recycle keratinous wastes. A group of proteolytic enzymes which are able to hydrolyze insoluble keratins more efficiently than other proteases are called keratinases (Onifade et al., 1998). They are produced by microorganisms like bacteria, fungi, actinomycetes etc.

The keratinolytic fungi for the study were isolated from hair baited soils. These strains were studied for the yield of keratinase by submerged and solid state fermentations. The fungal strain S125 which yielded highest amount of enzyme was identified as *Aspergillusflavus* by studying the cultural and morphological properties. The properties of purified extracellular keratinase produced by *Aspergillusflavus* S125 were studied and the enzyme was characterized. Experiments were done in triplicate.

MATERIALS AND METHODS

Effect of pH on enzyme activity

The effect of pH on the enzymatic activity of keratinase was investigated over a range of pH 7 to12 using buffers of strength 0.2M. The buffers used were Tris-HCl (pH 7-9.0), Phosphate buffer (pH 9.0-10.0) and Glycine-NaOH (pH 10.0-12.0). Reactions were carried out at 55˚C for 15 minutes.

Effect of temperature on enzyme activity

The effect of temperature on the enzyme activity was studied in Glycine-NaOH buffer 0.2M (pH 10.0) using keratin as the substrate. Keratinase was tested at different temperatures ranging from 30-70˚C. Reactions were carried for 15mins.

Effect of inhibitors on activity

The effect of different inhibitors on the activity of the enzymes was studied. The enzymes were incubated with various inhibitors at 10mM concentrations for 15 mins.at 55˚C and the residual activities were determined.

Effect of metal ions on activity

Effect of different metal ions on keratinase activity was analysed using 10 mM of Fe_2^+, Cu_2^+, Zn_2^+, Ca_2^+, Mg_2^+, Mn_2^+, Hg_2^+, Ba_2^+, Na^+ and K^+ in 20mM in Tris-HCl buffer at pH 9 and temperature 55˚C for 15 minutes.

Effect of pH on stability

pHstability of keratinase enzyme was investigated. The enzymes were incubated in buffers (0.2M) with different pH for 1 h at 55˚C. The buffers used were Tris-HCl (pH 7.0-9.0), Phosphate buffer (pH 9.0-10.0) and Glycine-NaOH (pH 10.0-12.0). Activities of the enzyme were determined before and after incubation. The percentage of activities remaining was calculated.

Effect of temperature on stability

Thermal stability was examined by exposure of the enzyme solution in Glycine-NaOH buffer (0.2 M, pH 10) to different temperature for 2 h after which the enzyme solution was cooled rapidly. Enzyme activities were determined before and after heat treatments. The percentage of activities remaining after the heat treatments were calculated.

Substrate concentration

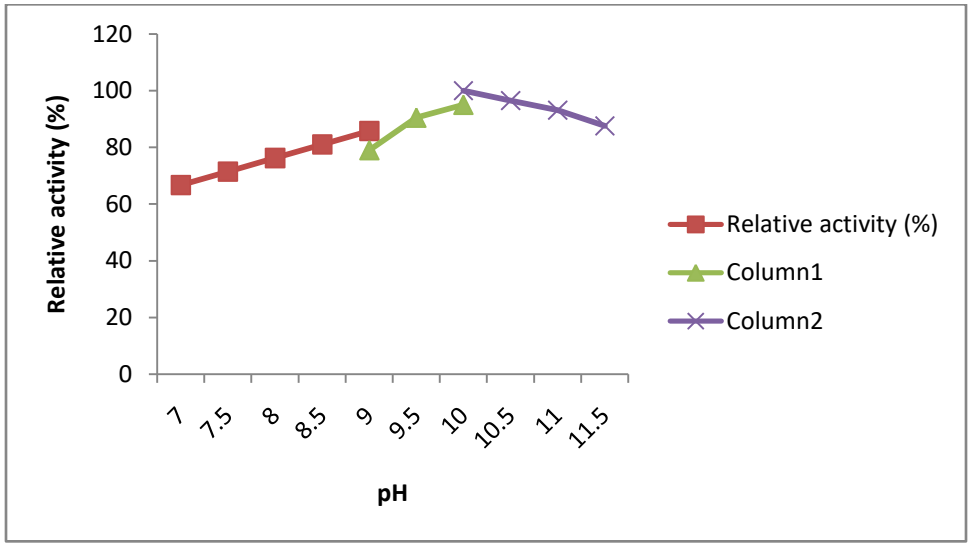
Kinetic studies were performed for keratinase activity using feather keratin as substrate and the data obtained is presented in LB plot. The Km and Vmax values for the keratinase were determined by using different concentrations of keratin added to assay system containing 0.5 mL enzyme at its optimum pH (10.5) and incubated at its optimum temperature (65˚ C) for 30 minutes. After that the velocity of enzyme was measured. LB plot between 1/S and 1/V was constructed to find out Vmax and Km.

RESULTS

Effect of pH on enzyme activity

Relative activities of the enzyme at different pH are shown in figure 1. The maximum activity shown by the enzyme is taken as 100%.

Figure 1 Effect of pH on enzyme activity



The optimum pH for degrading keratin by keratinase enzyme was 10.

Effect of temperature on enzyme activity

The relative activities of purified enzymes at different temperatures are shown in the figure 2 below. The maximum activity shown by the enzyme is taken as 100%.

Figure 2 Effect of temperature on enzyme activity

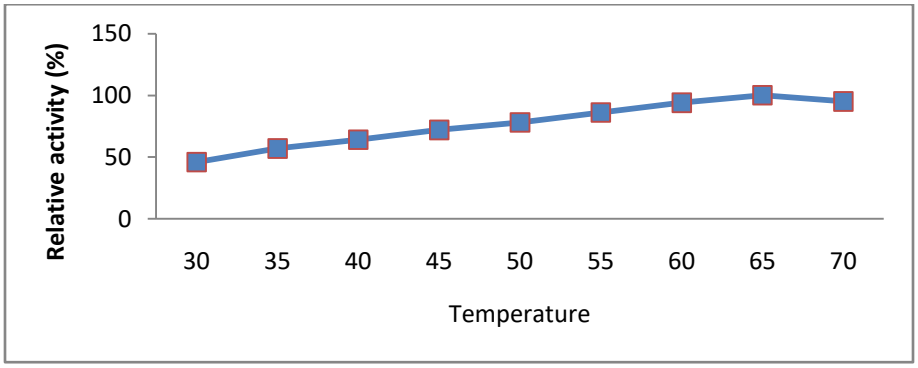


Figure 2 Effect of temperature on enzyme activity

The highest temperature for maximum keratinolytic activity by the enzyme was 65˚C.

Effect of inhibitors

The influence of different inhibitors on keratinase activity was investigated using phenyl methyl sulphonyl fluoride (PMSF), iodoacetic acid (IAA), $HgCl_2$, and ethylene diamine tetra acetate (EDTA) of 10 mM concentration each.

Results are shown in Table 1

Table 1 Effect of inhibitors on enzyme activity

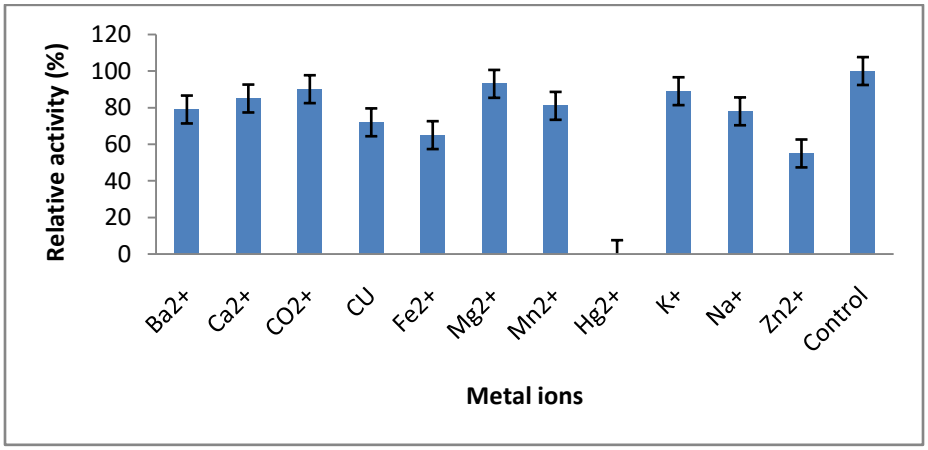
Inhibitors	Relative activity (%)
PMSF	4.6
IAA	97
EDTA	95.5
$HgCl_2$	0
Control	100

Total inhibition of enzyme activity was noted in presence of $HgCl_2$ and almost a negligible activity was observed with PMSF.

Effect of various metal ions on enzyme activity

The effect of various metal ions was tested. Results were shown in Fig.3. Activities are expressed relatively to the controls which are taken as 100%. It was found that none of the metal ions have enhancing effect on enzyme activity. Hg2+ exhibited a complete inhibitory effect. The activity. decreased in the presence of Cu , Fe++ and Zn2+ also.

Figure 3 Effect of metal ions on relative enzyme activity

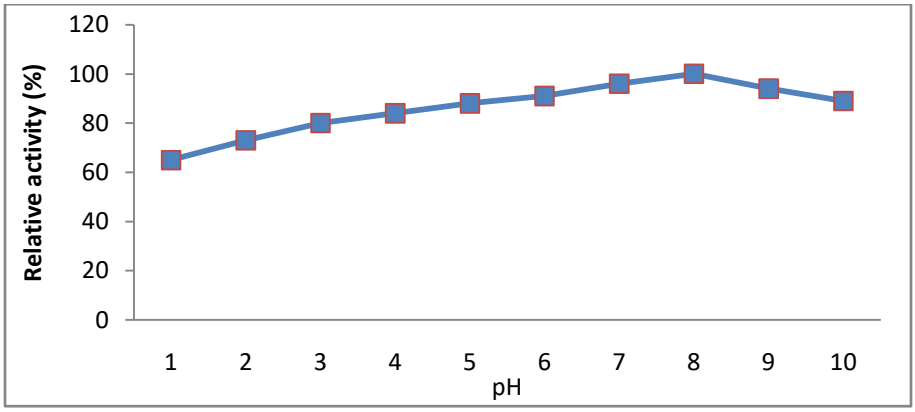


All the metal ions used presented the enzyme activity lesser than the control and mercury showed zero activity.

pH stability

pH stability of the enzyme was investigated. The percentage of activity remaining after incubation of enzyme in buffers with different pH is shown in the figure 4. The activity before incubation has been taken as 100%.

Figure 4 Stability profile of enzyme at different pH

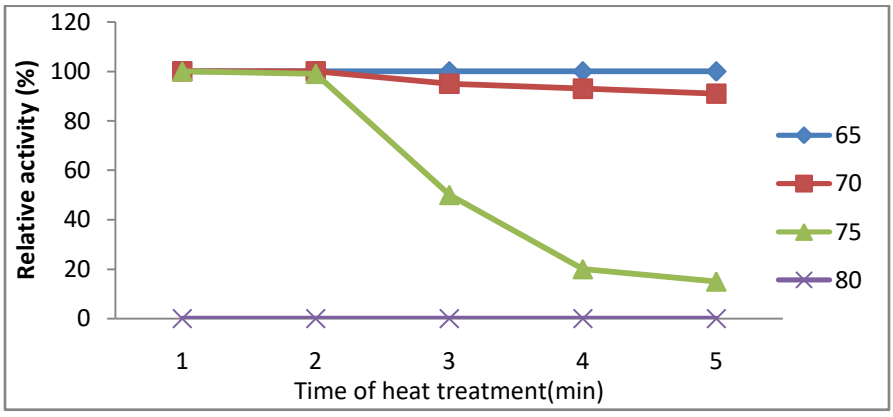


The enzyme maintained more than 80% of its original activity in the pH range 8-11.5

Temperatures stability

The results of stability profile of enzyme at various temperatures were shown in figure 5.

Figure 5 Stability profile of enzyme at various temperatures

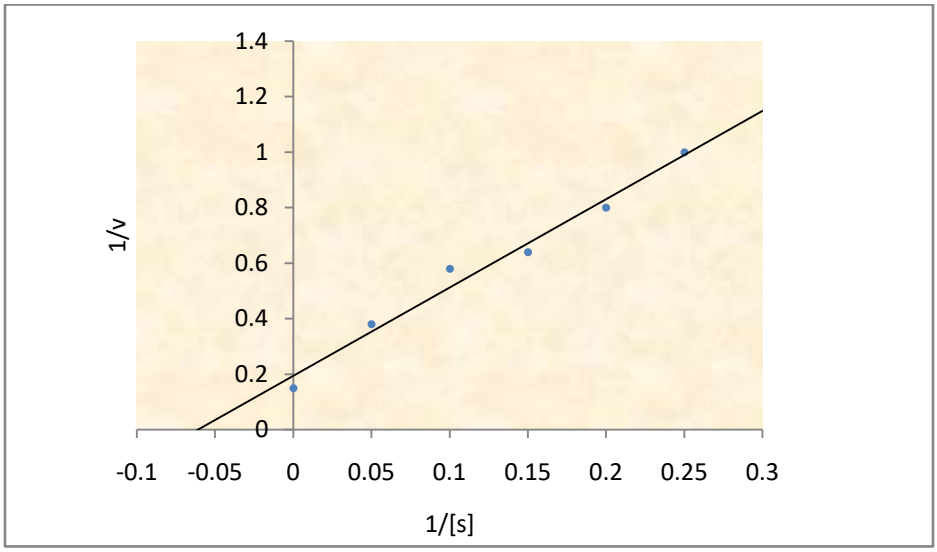


Thermal stability of the keratinase was investigated by heating the purified enzyme for 20 min at different temperatures in 0.2 mM Glycine-NaOH(pH 10.0). The enzyme was very stable up to 70℃ followed by a loss of activity above 70℃. The enzyme retained more than 90% and 15% of its activity at 70℃ and 75℃, respectively. However, the enzyme was completely inactivated at 80℃.

Substrate concentration

The Km value and V max calculated from the plot given in figure 4.9 were 15.38 mg/ml and 5U respectively.

Figure 6 LB plot of keratinase



DISCUSSION

Some of the properties of the keratinase enzyme purified from the culture supernatant of *Aspergillusflavus* S125 were studied. The optimum pH for keratinase activity obtained was 10.5. The enzyme activity study in *Cunninghamellaechinulata* showed better result in optimum pH 10. (More *et al*., (2013).

The present study revealed high keratinase activity at the temperature 65˚C. Almost similar temperature (60˚C) was observed in keratinolytic serine proteinase from *Streptomyces albidoflavus*(Bressollier,1999). Similarly active keratinase enzyme activity was noticed in *Paecilomyces marquandii* at temperature which was determined to be 60 to 65°C. (Gradisar,2005). Thanaa*et al.,* (2010) have shown that 70°C was the optimum temperature of enzyme activity in *Aspergillusoryzae*NRRL-447.

The effect of inhibitors on keratinase activity of purified enzyme from *Aspergillusflavus* 125 brought out the result that the enzyme was inhibited by Hg2 + and PMSF. Similar result was obtained in a study by More *et al*., (2013) in *Cunninghamellaechinulata*. Keratinase inhibition by

Hg2+ may suggest that a free cysteine is present at or near the active site (Lusterio,1992). EDTA and IAA displayed no effect on inhibiting the activity of the enzyme.

No significant effect on the enzyme activity was observed with metal ions and total inhibition of activity by Hg2+. It has been suggested that inhibition by Hg2+ 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. (Suntornsuk, 2005).

Experimenting the pH stability of the enzyme revealed that the enzyme maintained more than 80% of its original activity in the pH range of 8-11.5. Similar results were obtained by Korkmaz*et al*.(2004) which indicated that the *Bacillus licheniformis* enzyme was active over a wide range of pH with optimum 11.0. The alkaline serine-type keratinase from *A.Terreus*showed maximum activity at pH 8.5 (Chakarabarti*et al*., 2000). An overview of literature on pH stabilty indicates that keratinases are generally active and stable over a wide range of pH from 5 to 13. Keratinases from most bacteria, actinomycetes and fungi have pH optima in neutral to alkaline range. Enzyme with optimum activity at alkaline pH has definite advantage in application both in degradation of feather as well as in leather industry. The proteases active in the pH range of 8-12 and stable at alkaline pH are known as potential candidates for various commercial applications such as detergent production, dehairing of hides, and silver recovery from waste X-ray and photographic films (Rao *et al.,* 1998).

Thermal stability of the keratinase was investigated by heating the purified enzyme for 20 min at different temperatures in 0.2 M Glycine-NaOH(pH 10.0). The enzyme was very stable up to 70℃ followed by a loss of activity above 70℃. The enzyme retained more than 90% and 15% of its activity at 70℃ and 75℃, respectively. However, the enzyme was completely inactivated at 80℃. A study report on thermal stability of enzyme produced by *Streptomyces albus* AZA, the enzyme retained its complete stability for one hour at 70ºC. The high thermostability of the enzyme allows performance of the experiments at 65-70ºC which minimizes the risk of microbial contamination. (Mona, 2007). Some thermostablekeratinases have been found with the activity up to 90ºC.(Friedrich *et al*., 2003)

Kinetic studies were performed using feather keratin substrate. The affinity of keratinase enzyme to hydrolyse keratin substrate at a temperature 65˚C and pH 10.5 was

investigated. The Km value and V max calculated from the plot given were 15.38 mg/ml and 5U respectively.

It can be concluded that *A.flavus*is a potential source of keratinase possessing important properties like high pH and thermal stability and can be utilized in the degradation of feather as well as in leather industry. The proteases active in the pH range of 8-12 and stable at alkaline pH are known as potential candidates for various commercial applications such as detergent production, dehairing of hides, and silver recovery from waste X-ray photographic films etc.

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