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PURIFICATION AND CHARACTERISATION OF KERATINASE FROM *BACILLUS* SP. KP1

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ABSTRACT

In this study we purified and characterized a keratinase produced by a newly isolated bacterium *Bacillus* sp. KP1. The crude enzyme produced was purified by ammonium sulphate precipitation followed by DEAE Cellulose and Sepharose G-75 chromatography. Molecular mass of 30 kDa was determined by SDS-PAGE. Optimum pH and temperature for enzyme activity were found to be 8.0 and 50 °C respectively. Enzyme got inhibited by EDTA while retained its activity in the presence of PMSF and DTT indicating it's a metallo-enzyme. When effect of surfactants was studied on highest enzyme activity of 131± 3.9% was observed in the presence of triton X-100. Other surfactants also stimulated enzyme activity(triton X-100, tween-20, tween-80). When effect of metal ions (Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Hg^{+}, K^{+}, Na^{+}, Cu^{2+}, Fe^{3+}, Al^{3+}) was studied highest enzyme activity of 115 ± 2.8% was achieved for Ca^{2+} ions.

KEYWORDS: *Bacillus* sp. KP1, keratinase, ammonium sulphate precipitation, chromatography, surfactants, SDS-PAGE, metal ions.

INTRODUCTION

Keratinases (E.C.3.4.21/24/99) are a particular class of proteolytic enzymes that display the capability of degrading insoluble keratin substrates (Langeveld *et al.*, 2003).

Keratinases have a wide range of potential applications, including keratinous waste processing, food and feed supplements, detergent additives, and chemicals used in leather industries (Brandelli et al. 2010). A wide variety of microorganisms, including fungi and bacteria, can produce keratinases (Brandelli et al. 2010). Bacillus species such as *Bacillus pumilis* (Son et al. 2008), *Bacillus licheniformis* (Ni et al. 2011) and *Bacillus subtilis* are keratinase producers (Macedo et al. 2005). The objectives of the present work were to purify and characterize keratinase produced by a newly isolated *Bacillus* sp. KP1 form poultry waste.

MATERIALS AND METHODS

Keratinase production

Keratinase was produced form the previously isolated and characterized *Bacillus* sp. KP1. Bacterium was cultivated in 3.0 L bench top fermenter (Systronics India Ltd., Ahmedabad) with 1.5 L working volume. It was grown at 35°C, 150 rev min^{-1} and aeration was maintained at 1.0 L/min. Media used for production comprised (g l)1) NH4Cl, 0.5; NaCl, 0.5; KH2PO4, 0.4; K2HPO4, 0.3; MgCl2Æ6H2O, 0.24; yeast extract 0.1 and feather meal 10, at pH 7.0. After incubation of 120 h the crude enzyme was prepared by centrifugation at 4°C, 10,000x g for 10 min.

Keratinase assay

Keratinolytic activity was measured using the insoluble azokeratin as substrate, prepared according to Tomarelli et al. (1949). Assays were conducted for 30 min at 50 °C, incubating the sample (100 µL) with 500 µL of 1.5 % (w/v) azokeratin suspension in 50 mM phosphate buffer, pH 8.0. Reactions were stopped by adding 500 µL of a 30 % (v/v) trichloroacetic acid solution. Controls were prepared by adding the trichloroacetic acid solution immediately after the sample. Tubes were centrifuged at 10,000 rpm for 10 min and activity was measured at 440 nm. One unit of enzyme activity was defined as the amount of enzyme require to increase the solution absorbance by 0.1 per mL (U/mL) under the assay conditions.

Protein estimation

The protein was estimated by colorimetric method by Lowry et al., (1951) using bovine serum albumin as the standard protein.

Purification
The resultant supernatant was used as the starting material (crude enzyme) for keratinase purification. Supernatant was precipitated by the treatment of different concentrations of solid ammonium sulphate up to 80%. In each 100ml of supernatant solution, the individual concentration of ammonium sulphate was added pinch by pinch under ice cold conditions with continuous stirring at 300rpm on magnetic stirrer, then they were kept overnight in refrigerator at 4 °C. After overnight saturation, the supernatant solutions were centrifuged at 8500 x g for 40 min at 4 °C and the pellets were collected and dissolved in 10ml of 20 mM Tris-HCl buffer, pH 8.0 individually. Then the keratinase activity and protein contents of the dissolved materials were estimated. Fraction showing the maximum keratinase activity and protein content was used for further purification process.

Dialysis
Dialysis of the precipitate was done using cellulose tubing (molecular weight cutoff: 12−14 kDa). Dialysis bag was activated before. Glycerol was removed by washing the tube under tap water for 3h. Sulfur compounds were removed by treating the tubing with a 0.3% (w/v) solution of sodium sulphide at 80 °C for 1 min. Again, the bag was washed with hot water (60 °C) for 2 min, followed by acidification with a 0.2% (v/v) solution of sulfuric acid, and was rinsed with hot water to remove the acid traces. The activated dialysis bag was filled with resuspended solution from the previous step and both the sides were sealed without any air bubbles. The bag was kept in a container filled with 20 mM Tris-HCL buffer (pH 8.0) and incubated overnight. The unit was placed on the magnetic stirrer and subjected to stirring. The buffer was changed every 3 h. Dialysis was performed overnight. Later the dialysis bag was punctured and the sample was collected for further purification and analysis.

Ion exchange chromatography
Dialyzed sample was applied to a column of DEAE-cellulose (0.5cm x 20cm), which was pre-equilibrated with 20 mM Tris-HCl buffer at pH 8.0. The enzyme sample was allowed to bind to the gel for 1 h at 4 °C. After this buffer was added slowly and allowed to flow through the column to remove unbound protein fractions (10 ml at a flow rate of 1 ml min^{-1}). Then bound protein fractions were eluted with a linear gradient of NaCl (0.05 to 0.5M made in Tris-HCl buffer of pH 8.0). Different fractions were collected at a flow rate of 1 ml min^{-1} in 3 ml fractions and were assayed for keratinase activity and protein content. Fractions showing high activity were pooled together and used for further purification.

Gel filtration chromatography
Fractions showing highest enzyme activity were pooled and used for further purification by gel filtration using Sephadex G-75 column (1.5cm x 40cm). Twenty mM Tris-HCl buffer (pH 8.0) was used for elution. Three ml fractions were collected and fractions showing highest enzyme activity were pooled together for further studies.

Molecular mass determination
The molecular weight and purity of lipase was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as per the method of Laemmli (1970) on a vertical slab gel (GeNei™, Bangalore). Standard protein markers-Phosphorylase b (97.4kDa), Bovine serum albumin (66 kDa), Ovalbumin (43 kDa), Carbonic anhydrase (29 kDa), Soybean trypsin inhibitor (20.1 kDa), and Lysozyme (14.3 kDa) were used. Molecular weight of keratinase was determined by comparing it with the relative mobility of the standard marker proteins.

Effect of pH and temperature
Optimum pH for the enzyme activity was determined across the range of 4-11 by using following buffer solutions citrate phosphate buffer (pH 4-6), Tris-HCl buffer (pH7-9) and glycine-NaOH buffer (pH 10-11).

The optimal temperature for the purified enzyme activity was investigated by performing the enzyme reaction at different temperatures from 30℃ to 80℃ in 50 mM phosphate buffer (pH 8.0).

Effects of metal ions, inhibitors and surfactants
The enzyme was treated with the following metal ions Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Hg^{+}, K^{+}, Na^{+}, Cu^{2+}, Fe^{3+}, Al^{3+} at a concentration of 5 mM. Protease inhibitors: phenylmethylsulphonyl fluoride (PMSF), Ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT) at a concentration of 5 mM and following surfactants Sodium dodecyl sulphate (SDS) (5 mM), triton X-100, tween-20 and tween-80 at a concentration of 1% except SDS. Keratinase was pre-incubated with surfactants, inhibitors and metals at 50 °C for 30 min before residual keratinolytic activity was measured. Keratinolytic activity in the absence of surfactants, inhibitors and metallic ions was considered as 100 % of activity.

RESULTS AND DISCUSSION

Purification of keratinase: Specific activity of crude enzyme was 2.10 U/mg. After ammonium salt precipitation specific activity of 3.6 U/mg was obtained. While after ion exchange chromatography specific activity of 2.53 U/mg was achieved and after final purification by gel exclusion chromatography specific activity of 7.33 U/mg and purification of 3.49-fold was achieved with 72.13% recovery (**Table 01**).

Table 01: Purification steps for keratinase.

	Activity (U/mL)	Protein (mg/mL)	Specific activity (U/mg)	Purification fold (%)
Crude	6.1	2.9	2.10	100
Ammonium sulphate precipitation (80%)	5.2	1.7	3.06	1.46
DEAE-Cellulose	4.8	0.9	5.33	2.53
Sephadex G-75	4.4	0.6	7.33	3.49

Molecular mass determination by SDS-PAGE
Molecular mass and purity of purified keratinase was confirmed by SDS-PAGE. A single band of molecular mass 30 kDa was observed (**Figure 01**). Prakash et al. (2010) reported that the molecular mass of keratinases generally ranges from 30 to 66 kDa. Linn et al. (1992) isolated keratinase form *B. licheniformis* PWD-1 which had a molecular mass of 33 kDa. In another study Suh and Lee (2001) isolated keratinase from *B. subtilis* KS-1 with a molecular mass of 25.4 kDa.

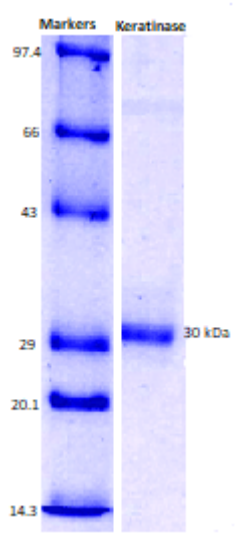

Figure 1: SDS PAGE.

Effect of pH and temperature on keratinase activity
Effect of pH and temperature on enzyme activity was observed using azokeratin as substrate. The keratinolytic activity was assayed in the pH range of 4-11 using buffers of different pH values at 50 °C. The activity obtained at pH 8 was taken as 100 %. Optimum enzyme activity was observed at pH 8.0 (**Figure 02**). Previously reported keratinases are active over a range of pH from 5-13. The least reported optimum value of pH 5 was found for keratinase from *Streptomyces pactum* DSM40530 (Bockle et al. 1995) and the highest optima of pH 13 was observed for *Bacillus halodurans* AH-101 (Takami et al. 1999). However, most of the other keratinases isolated have optimum activity in neutral to alkaline range (Bockle et al. 1995; Dozie et al. 1994). Enzyme with optimum activity at alkaline pH, has definite advantage in application both in degradation of feather as well as in leather industry as significant increase in pH are found associated in the processes.

The keratinolytic activity was assayed at temperatures between 30 °C and 80 °C in phosphate sodium buffer 50 mM, pH 8.0. Optimum relative activity was observed at 50 °C (**Figure 03**). Keratinolytic proteases from variable bacterial strains have been reported to display temperature optima between 40 °C and 80 °C (Verma et al. 2017) for example keratinase from *B. pseudofirmus* AL-89 had optimum temperature range of 60-70 °C (Gessesse et al., 2003), *Nocardiopsis sp.* TOA-1 had optimum temperature of 60 °C (Mitsuiki et al., 2004), and a few have exceptionally high temperature optimum of 100 °C (Nam et al., 2002).

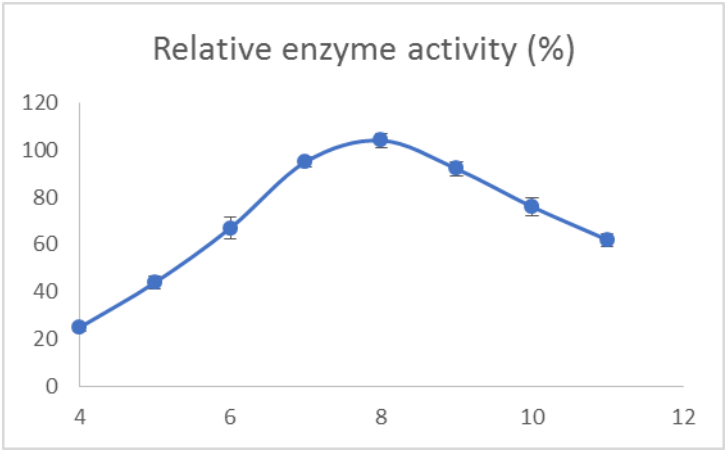

Figure 2: Effect of pH on keratinase activity.

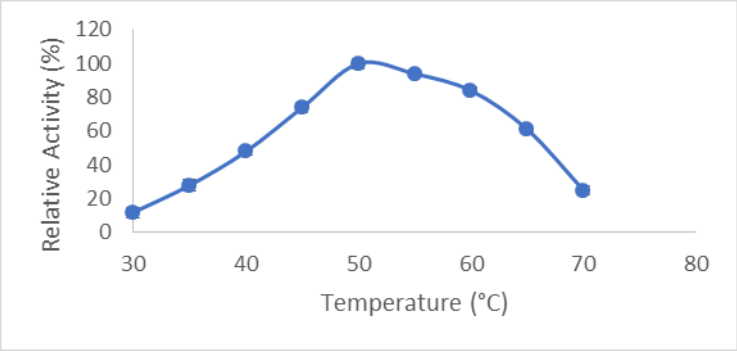

Figure 3: Effect of temperature on keratinase activity.

Effect of metal ions, inhibitors and surfactants

Studies on the effect of metal ions on keratinase activity revealed that the enzyme was mostly active and stable in the presence of various metal ions. Optimum activity was observed with calcium ions while least activity was observed with sodium ions. Keratinase was significantly stable in the presence of serine protease inhibitor PMSF and DTT with residual activity of 88 ± 3.2% and 92 ± 3.6% respectively. Keratinase lost its complete activity in the presence of EDTA (chelating agent). Metal ion chelators remove metal cofactors that assist in the maintenance of enzyme catalytic activity, hence promote loss of enzyme–substrate affinity and/or structural destabilization. This indicates that the keratinase is metallo-protease. The sensitivity of proteases to a different class of inhibitors is used to classify them, and comprehensive reports have described keratinases to predominantly belong to serine or metallo class of protease (Brandelli et al; 2010). Keratinase activity was promoted in the presence of SDS, triton X-100, tween-80 and tween-20. In similar studies Riffel et al. (2007) reported metallo-keratinase from *Chryseobacterium* sp. kr6 with significant stability in the presence of 2-mercaptoethanol and triton X-100. Nnolim et al. (2020) also reported significant stability of keratinase from *Bacillus* sp. Nnolim-K1 in the presence of triton X-100 and tween 80 (**Table 02**).

Table 2: Effect of metal ions, inhibitors and surfactants on keratinase activity.

Metal ion	Concentration	Relative activity (%)
Ca^{2+}	5 mM	115 ± 2.8
Mg^{2+}	5 mM	94 ± 3.1
Mn^{2+}	5 mM	88 ± 3.1
Zn^{2+}	5 mM	81± 1.4
Hg^{+}	5 mM	80± 1.9
K^{+}	5 mM	91± 3.2
Na^{+}	5 mM	69± 1.2
Cu^{2+}	5 mM	70 ± 3.1
Fe^{3+}	5 mM	89± 2.5
Al^{3+}	5 mM	82± 3.2
Protease inhibitors		
PMSF	5 mM	88± 3.2
EDTA	5 mM	0.0
DTT	5 mM	92± 3.6
Surfactants		
SDS	5 mM	107± 2.8
Triton X-100	1%	131± 3.9
Tween-20	1%	109± 2.1
Tween-80	1%	121± 3.8

CONCLUSION

Keratinase isolated form *Bacillus* sp. KP1 had a molecular mass of 30 kDa. Keratinase got inactivated in the presence of EDTA which shows that the enzyme is metallo-keratinase. The enzyme was able to retain most of its activity in the presence of metal ions and protease inhibitors i.e., PMSF and DTT. Surfactants i.e., SDS, triton X-100, tween-20 and tween-80 promoted enzyme activity. Optimum activity of enzyme was shown at pH 8.0 and temperature 50 °C. All the results signify the robust nature of the isolated enzyme and its potential for further scale-up studies and industrial use.

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