

Research Article

Isolation of *Chrysosporium indicum* from poultry soil for keratinase enzyme, its purification and partial characterization

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Abstract

Keratinases are produced by microorganisms as fungi, actinomycetes and bacteria and have the capacity to degrade tough insoluble keratin proteins, including feathers. Feathers are waste produced from the poultry industry worldwide and accumulated as solid waste. Therefore, keratinolytic fungal strains *Chrysosporium indicum* were isolated by hair baiting method from poultry farm soil of Punjab, India. Isolated *C. indicum* were screened for proteolytic activity on skimmed milk agar. Field Emission Scanning Electron Microscopy (FeSEM) analysis confirmed morphological characters as *C. indicum*. Fourier transform infrared spectroscopy analysis was studied for the structural and mechanism analysis of feather degraded during keratinase production. Keratinase enzyme was purified 48.03% recovery by ammonium sulphate precipitation, dialysis for desalting and chromatography. Diethylaminoethyl sepharose (DEAE sepharose) and Sephadex-G75 column were used to perform chromatography and partial characterization of the keratinase for temperature, pH, and substrate. The maximum keratinase activity was observed at 50°C, at pH 10. The maximum enzyme activity of 289.1 U/ml was observed with keratin powder as substrate and minimum enzyme activity 67.1 U/ml with keratin azure. This is the first report on the purification and characterization of keratinase by *C. indicum* using DEAE sepharose as affinity chromatography for the purification of the keratinase enzyme.

Keywords: *Chrysosporium indicum*, Fourier transform infrared spectroscopy, Keratinase, Purification, Scanning electron microscopy

INTRODUCTION

Keratins are insoluble molecules that comprise long polypeptide chains resistant to non-substrate specific proteases (Allpress *et al.*, 2002). Keratins belong to the scleroprotein group of fibrous family, which is found in feather, wool, horn nails (Kumar *et al.*, 2021). Keratinases are serine or metalloproteases proficient of degrading the structure forming keratinous proteins which cannot be solubilized completely by the purified keratinases (Ramnani *et al.*, 2005). Keratinase produced by keratin degrading microorganism as bacteria, actinomycetes and fungi (Kumar and Kushwaha, 2014). Several reports are available on isolation and screening of microorganism for keratinase production *Streptomyces* (Ya-peng *et al.*, 2007), fungi (Kumar and Kushwaha 2014). Optimization of growth media and cultural condi-

tions by bacteria *Bacillus licheniformis* was studied (Gupta and Ramnani, 2004, Cai *et al.*, 2008, Kainoor and Naik 2010), *Bacillus* sp. (Kanchana *et al.*, 2012), (Mehta *et al.*, 2014), *Acremonium strictum* (Kumar and Kushwaha 2012). Few reports are available on purification and characterization of keratinase enzyme produced by microorganisms *Lysobacter* sp. (Allpress *et al.*, 2002), *Bacillus licheniformis* (Suntornsuk *et al.*, 2005), *Bacillus thrungensis* (Sivakumar 2012), *Streptomyces albus* (Esawy 2007), *Microsporium gypseum* (Raju *et al.*, 2007), *Trichophyton simmi* (Singh 1997), *Aspergillus parasiticus* (Anitha and Palanivelu, 2012), *Cunninghamella echinulata* (More *et al.*, 2013). However most of the study belong to bacterial isolates or dermatophytes, therefore the purification and characterization of keratinase from nonpathogenic microorganisms is important because of their capacity to degrade the ker-

atin and various application. Keratinase could be useful into development of economically feather meal (Onifade *et al.*, 1998, Lin *et al.*, 1999), nitrogenous fertilizer, biodegradable films, glues foils (Friedrich and Antranikian 1996, Schrooyen *et al.*, 2001, De Toni *et al.*, 2002, Gupta and Ramnani 2006), prion degradation (Langeveld *et al.*, 2003), feather compost for plant growth enhancement (Kumari and Kumar 2020, Kumar *et al.*, 2020). The objective of the present study was to isolate a potent keratinolytic fungi and to study the purification and characterization of its keratinase enzyme.

MATERIALS AND METHODS

Isolation and screening and identification of Fungi

Keratinophilic fungi were isolated by the hair baiting method of Benedek (1962). Soil samples were taken from a poultry farm in Chunni Kalan, Punjab, and placed in Petri plates with sterile water to moisten them. Baits made of chicken feathers were used.

For the primary screening of proteolytic activity, 5 g L⁻¹ peptones, 3 g L⁻¹ yeast extract, 100 mL L⁻¹, sterile UHT nonfat milk, and 12 g L⁻¹ agar were used following the method of Riffel and Brandelli (2006). The Fungi were inoculated on skimmed milk agar plates and incubated for 3, 6, 9 and 12 days for 28°C to observed keratinolytic potential. The diameter of the clearing zone was measured to quantify keratinase enzyme activity.

Morphology of *Chrysosporium indicum*

C. indicum was observed by the morphological characters on agar media petri plates simple microscopy in the laboratory. Field Emission Scanning Electron Microscopy (FeSEM) of *C. indicum* at Central instrumentation facility (CIF), Lovely Professional University, Jalandhar (Pb) having a resolution as 1 Micrometer and particle of the size of 100nm.

FTIR analysis of feather degradation

Feather degradation by *C. indicum* was studied by FTIR performance which was recorded by using Perkin Elmer spectrum having a resolution of cm⁻¹ and scan range 4000 cm⁻¹ to 250 cm⁻¹ in CIF Lovely Professional University Punjab.

Keratinase production

Keratinase production media was prepared by the method of Kumar and Kushwaha (2012) by taking 5g feather, 2g glucose, 5g peptone, 5g yeast extract, 1g K₂HPO₄, 3g KH₂PO₄, 1g CaCl₂ and 1g MgSO₄ dissolved in 1000ml. The above media was inoculated with a disc of 8 days old culture of *C. indicum*. fermentation was carried out at 28±2 °C and 200 rpm on a rotary shaker incubator for 12 days. After incubation, filtrates were obtained and used for enzyme assay.

Enzyme assay

Keratinase production was measured by the method of Gupta and Ramnani (2004). The test tube containing 1 ml of enzyme 4 ml of glycine-NaOH buffer and 20 mg of chicken feather (collected from poultry shop) were incubated at 60°C for 60 min. 4ml of 5% (w/v) TCA were added to terminate the reaction and incubated 60 min at room temperature. Feathers were removed by filtration. Control was prepared similarly except 1ml of 5% TCA and 3ml of Buffer were added. Keratinase activity was recorded at 280 nm against the control using UV-VIS spectrophotometer. An increase 0.01 in absorbance was considered as 1 unit of enzyme activity per ml.

Purification of enzyme

Ammonium sulphate precipitation

The enzyme was purified by the method of Suntornsuk *et al.* (2005) and Shivakumar *et al.*, (2012). 0-20% (5.49 g) ammonium sulphate was added to 50 ml of crude enzyme. After precipitation, the pellet was collected and centrifuged at 5000 rpm for 25 min. in cooling centrifuge. The subsequent saturation was done 20-40 %, 40-60-%, 60-80%, 80-100%. The precipitates were dissolved in 0.05M glycine-NaOH buffer (pH 10). After precipitation enzyme was desalted by using Himedia dialysis membrane-50. Dialysis membrane was regenerated by dipping in a solution of 2% sodium bicarbonate solution and 0.5% EDTA boiled for 10 min. after filling the enzyme membrane was left in glycine NaOH buffer for 3-4 hours for desalting.

Gel filtration chromatography

Dialyzed enzyme was purified with Sephadex G-75 (mol. wt 1000-5000) column chromatography. The enzyme solution was run into column according to 6ml/hr flow rate with .05M glycine NaOH (pH 10) buffer and fractions of 3ml were collected. The absorbance of the fractions was observed at 280nm.

Affinity chromatography

The collected fraction was applied to diethylaminoethyl (DEAE) Sepharose column. Enzyme was run into a column according to 6ml/hr flow rate with 0.05 M glycine-NaOH buffer (pH 10). The column was eluted with 0.025M glycine-NaOH buffer (pH 7 with flow rate 12ml/hr. Fraction of 3ml was collected and absorbance was recorded at 280nm.

Enzyme characterization

Temperature characterization

An enzyme assay was done at various temperatures to find the optimum temperature for keratinase activity. The temperature should be between 30 and 70°C at pH 10. The Keratinase assay conditions were same as enzyme assay.

pH characterization

Using 0.1 M phosphate buffer (6-8 pH) and 0.05M glycine-NaOH (pH 9-11), the effect of pH on keratinase was determined. The other cultural conditions were the same as enzyme assay.

Substrate characterization

The substrate BSA, feather, keratin powder, keratin azure were added 20 mg in each test and enzyme assay was performed.

RESULTS AND DISCUSSION

Isolation and screening of keratinophilic fungi

The keratinophilic fungi grown on the feather is shown in Fig 1 a and b. The Isolate (1026) keratinophilic fungi was identified as *C. indicum*. It was deposited in the Departmental laboratory of Dolphin PG College of Science & Agriculture, Chunnikalan (Pb) as JK14 and sent to National Fungal Culture Collection of India, Pune accession number. *C. indicum* showed the clearing zone tested on skimmed milk agar plates for its keratinase enzyme activity (Fig. 1c).

C. indicum attained 35-40 mm diameter growth in 8 days on PDA, cultured in 99mm Petri plates. Powdery white thin denser and up to 2mm high at the center, margin defined, regular, fimbriate, reverse cream coloured (Fig. 2a). Hyphae were hyaline, smooth and thin-walled and rarely branched, while sterile more frequently branched, when aerial hyphae fertile and infertile, 1.5-5.0µm wide, submerged hyphae mostly sterile, straight 1-4µm wide, the narrower often contorted. Terminal and lateral conidia sessile or on short protrusions of slightly swollen lateral branches, solitary, infrequently subtended by alternative conidium, subhyaline smooth or slightly echinulate, thin walled, ovoid to ellipsoid, 1 celled 4.0-7.0×2.0-3.0µm, with fairly broad basal scar (1-1.5 µm). Intercalary conidia less abundant, solitary sub hyaline (Fig. 2 b-h).

FTIR analysis

The feathers were degraded by *C. indicum* in submerged state fermentation (Fig. 4). Degraded feathers showed the presence of COOH and NH₂ group in FTIR analysis. After comparison of samples, it was found that the characteristic peaks were similar to each other and comparable with other studies in the case of keratin extraction from the chicken feather waste by application of reducing agent L-cysteine (Ma *et al.*, 2016). Band region found between 3500-3200 cm⁻¹ was attributed to the stretching vibration of -O-H- and -N-H amide band (Pavia *et al.*, 2008) and appeared in the range between 3000-2800 were related to symmetrical CH₃ stretching vibration. The strong band was attributed to -CO- stretching (amide I), which occurred in the range of 1700-1600 cm⁻¹ (Mohanty *et al.*, 2005). The band (amide II) in the range of 1580-1480 cm⁻¹ was for -N-H banding and C-H stretching (Eslahi *et al.*, 2013). The band associated with amide III observed weak between 1300-1220 cm⁻¹ which was derived from C-N stretching C-O- bond band and C-C stretching (Vasconcelos *et al.*, 2008). In the sulphoxide region at 1073 cm⁻¹ the band corresponding to S-O was observed for degraded feathers (Fig. 3). Untreated feather had a band with low intensity and was poorly visible (Kumar and Yadav, 2020), while in degraded feather, the intensity of the band was high. The band between 750-600 cm⁻¹ was related to -N-H out of plane banding.

Characterization of purified enzyme

Temperature characterization

The enzyme after partial purification was subjected to enzyme assay. The purified enzyme by Sephadex G-7 column and DEAE sepharose column showed maximum enzyme activity of 69.2 U/ml at temperature 50°C and 61.9 U/ml was observed at temperature 60°C (Fig. 5 & 6).

pH characterization

Enzyme assay of Sephadex G-75 and DEAE cellulose column purified enzyme done for pH characterization at



Fig. 1 a. Growth of *C. indicum* on feather baits, **b.** *C. indicum* degraded feather, **c.** clearing zone by produced by *C. indicum* on skimmed milk agar.

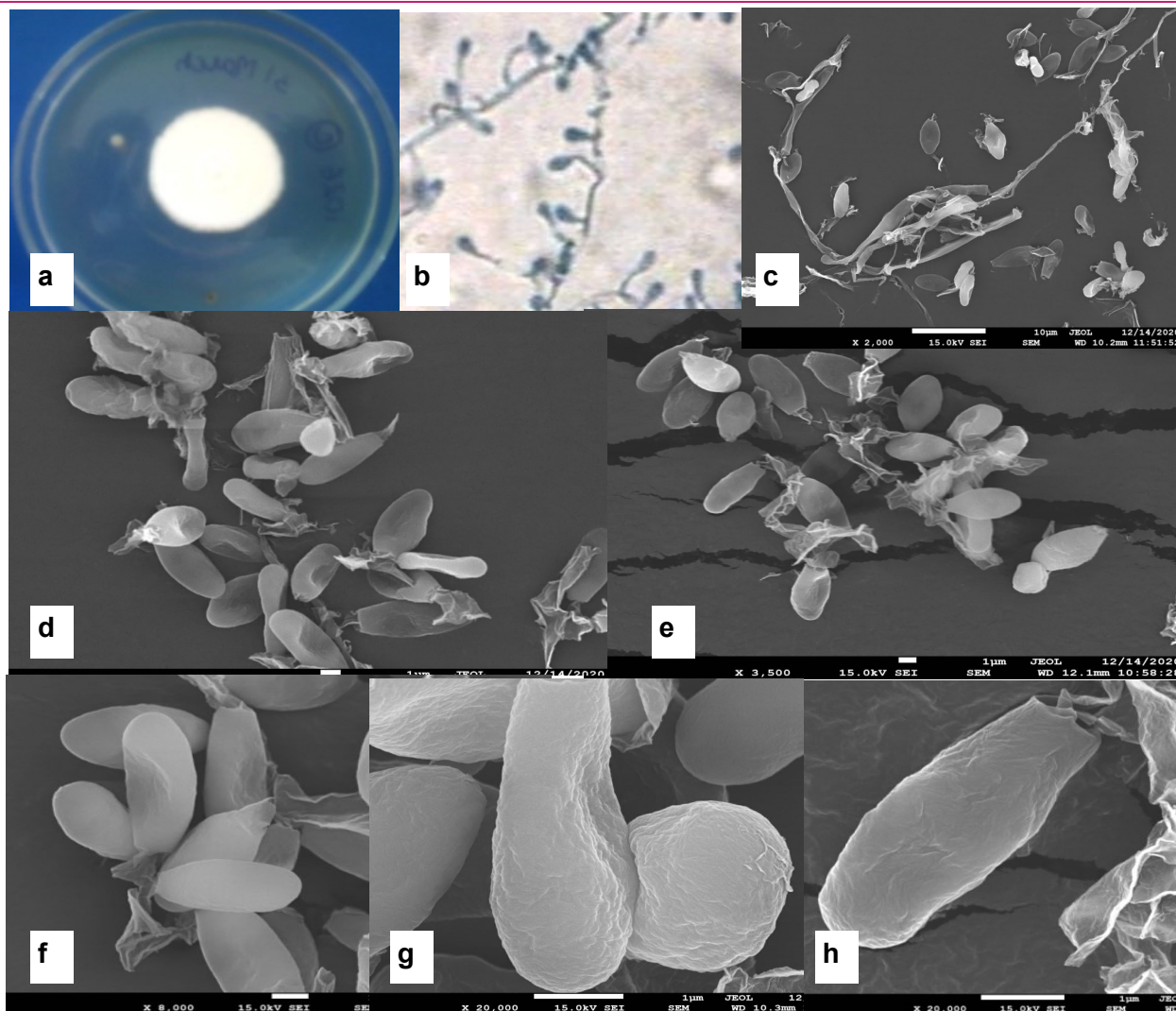


Fig. 2. a. Growth of *C. indicum* on Potato Dextrose Agar, b. *Conidia* 1000X, c. SEM of *C. indicum* Hyphae & terminal conidia (10 μm 2000X). d-e. 1 μm 3500X, f. 1 μm 10000X g-h. *Conidia* 1 μm 20000X.

incubation temperature of 50°C. showed that maximum enzyme activity of 69.2 U/ml was at pH 10 and 61.9 U/ml was at pH 10 respectively (Fig.7 and 8).

Substrate characterization

The maximum enzyme activity of 289.1 U/ml was observed with keratin powder as substrate and minimum enzyme activity 67.1 U/ml was observed with keratin azure with enzyme purified with gel filtration. The maximum enzyme activity of 262.9 U/ml was observed with keratin powder and minimum enzyme activity 56.5 U/ml was observed with keratin azure with enzyme purified with affinity chromatography (Fig. 9 A & B).

Comparison of keratinolytic activities was difficult due to different conditions applied during experimentation as keratin substrate variety, diverse chromatography column and microorganism used in study. Gradisar *et al.*, (2005) compared optimum pH for keratinases activity from *Paecilomyces marquandii*, *Doratomyces microspores* and *A. flavus* and recorded pH 8, 8 and 11 re-

spectively. Kim (2007) studied *Aspergillus fumigatus* and purified keratinase gave maximum activity at pH 8 and temp 45°C. However, enzyme keratinase was active at 30-70°C temperature and at pH 7-10. Vigneshwaran *et al.* (2010) reported activity of

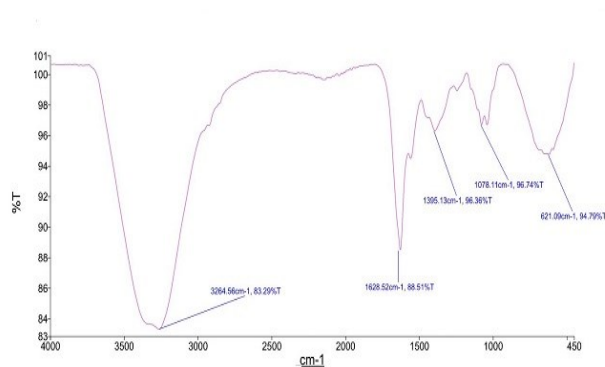


Fig. 3. FTIR spectra of *C. indicum* degraded feather.



Fig. 4. Feather degradation in submerged state fermentation (Control & Test).

keratinase enzyme from *Bacillus licheniformis* was characterized for optimum pH 7, temp 60°C. Nayaka and Vidyasagar (2013) observed pH 7 as optimum for keratinase activity from *Streptomyces albus* while *B. megaterium* observed maximum keratinase activity at optimum 9.0 pH and 50°C temperature (Saibabu *et al.*, 2013). Poopathi *et al.*, (2014) obtained 2.3-time purifi-

cation in ammonium sulphate while 11.68-fold with sephacryl S-200 column while in present study 48.03% recovery of enzyme was recorded after DEAE sepharose column chromatography which was slightly higher than 30% observed by Lee *et al.* (1987) for *Microsporium canis*. Veerapura *et al.* (2019) observed optimum keratinase activity at pH 7 and temperature of 35°C by *Serratia* sp. Hamiche *et al.* (2019) obtained keratinase enzyme KERZT-A was ideally dynamic at pH 6.5 and 50°C, KERZT-B appeared ideal at pH 8 and 60°C. The keratinase from *Bacillus* sp. showed optimum activity at pH8 and 60-80°C temperature (Nnolim and Nwodo, 2020). Results obtained in the present study were in accordance with the study of Gradisar *et al.* (2005) and Kim (2007) Saibabu *et al.* (2013) as optimum of pH 10 and temperature of 50-60°C and were in range. There was always some variation due to bacterial and fungal isolates used for enzyme production. In present study, keratin powder substrate showed maximum enzyme activity of 289.1 U/ml as substrate while using a feather as substrate enzyme activity showed 61.9 U/ml. It was observed that the small size of the keratinous substrate was better for enzyme activity due

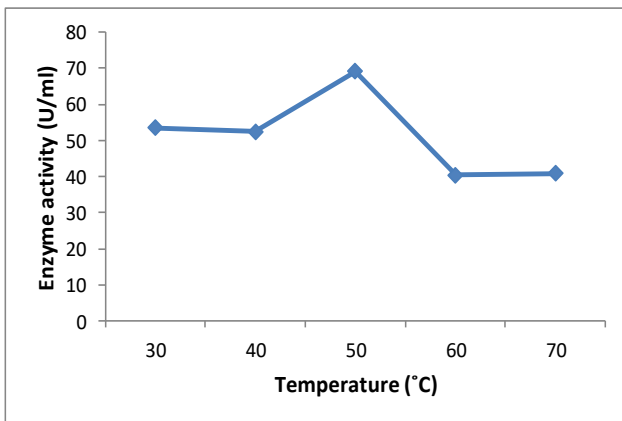


Fig. 5. Temperature activity of pure keratinase Sephadex G-75.

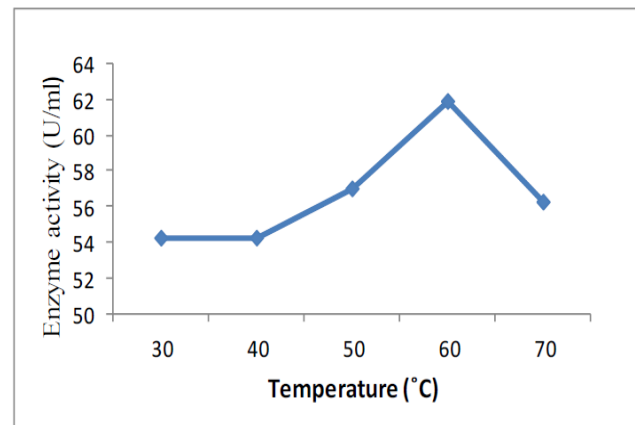


Fig. 6. Temperature activity of enzyme obtained from DEAE Sepharose column.

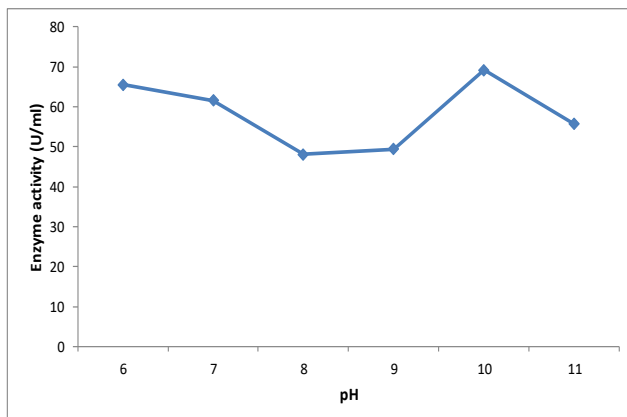


Fig. 7. pH activity for enzyme obtained from Sephadex G-75

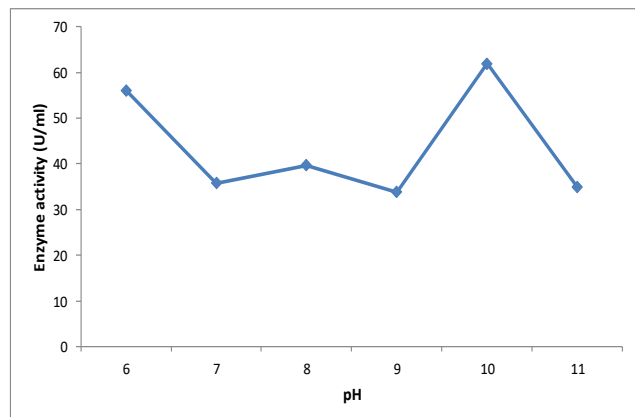


Fig. 8. Enzyme activity at pH purified by DEAE Sepharose column.

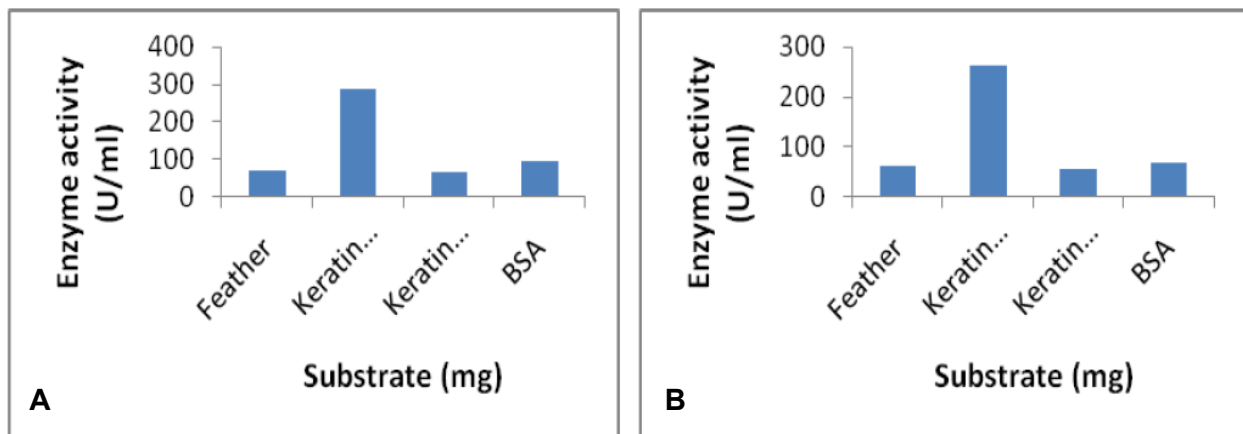


Fig. 9 A & B. Showing substrate activity (A. After Sephadex G-75, B. After DEAE Sepharose column).

to the powder state of matter always provide more surface area for enzyme-substrate reactions.

Conclusion

The isolate of *C. indicum* was found to be the most promising isolate for keratinase production and feather degradation. The maximum keratinase activity was observed at 50°C, at pH 10. Purified keratinase enzyme degraded a diverse substrate as keratin powder, keratin azure and feathers, BSA protein although the maximum activity 289.1 U/ml with keratin powder indicate that small substrate size is optimum for maximum degradation. The fungus *C. indicum* could be used to produce the enzyme keratinase, which has potential application in prion degradation, enhancing the nutritional value of animal feed containing keratin, development of keratin waste biofertilizers and keratin waste utilization.

Conflict of interest

The authors declare that they have no conflict of interest.

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